J Dai, Ph.D.

AD_____

Award Number: DAMD17-99-1-9530

TITLE: Neutral Endopeptidase Inhibits Neuropeptide Mediated
Growth of Androgen-Independent Prostate Cancer

PRINCIPAL INVESTIGATOR: Jie Dai, Ph.D.

CONTRACTING ORGANIZATION: Weill Medical College of
Cornell University
New York, New York 10021

REPORT DATE: September 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

1

20010327 089

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE September 2000	3. REPORT TYPE AND DATES COVERED Annual Summary (16 Aug 99 - 15 Aug 00)

4. TITLE AND SUBTITLE
Neutral Endopeptidase Inhibits Neuropeptide Mediated!
Growth of Androgen-Independent Prostate Cancer

5. FUNDING NUMBERS
DAMD17-99-1-9530

6. AUTHOR(S)
Jie Dai, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Weill Medical College of Cornell University
New York, New York 10021

E-MAIL:
jdai@mail.med.cornell.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited	12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The objective of this project is to elucidate the involvement of neutral endopeptidase (NEP), a cell-surface peptidase which inactivates active peptides and reduces local concentrations of peptide available for receptor binding and signal transduction, in the growth inhibition of androgen-independent (AI) prostate cancer. During the first year of the fellowship, we have for the first time shown that overexpression of cell-surface NEP induces growth inhibition and apoptosis in AI-prostate cancer. This growth inhibition induced by NEP results from PC cells arresting in a G1 phase arrest of the cell cycle and leading to increased expression of p21 and dephosphorylated Rb protein. Induction of NEP can inhibit the tumorigenicity by using the orthotopic model of prostate cancer in which PC cells are directly injected into the prostate. NEP is also involved in integrin mediated signaling pathways. Recombinant NEP and induced NEP can inhibit the bombesin and endothelin-1 stimulated FAK phosphorylation and cell migration. These studies will help delineate biological significance of NEP loss in the development and maintenance of androgen-independent prostate cancer, and indicate the potential for recombinant NEP or NEP directed gene therapy as therapy in patients AI PC.

14. SUBJECT TERMS Prostate Cancer	15. NUMBER OF PAGES 110
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Introduction

Neutral endopeptidase 24.11 (neprilysin, enkephalinase, CD10, EC 3.4.24.11) is a 90-110 kD cell-surface metallopeptidase which is normally expressed by numerous tissues, including prostate, kidney, intestine, endometrium, adrenal glands and lung. This enzyme cleaves peptide bonds on the amino side of hydrophobic amino acids and inactivates a variety of physiologically active peptides, including atrial natriuretic factor, substance P, bradykinin, oxytocin, Leu- and Met-enkephalins, neurotensin, bombesin, endothelin-1, and bombesin-like peptides. NEP reduces the local concentration of peptide available for receptor binding and signal transduction. Loss or decreases in NEP expression have been reported in a variety of malignancies, including renal cancer, invasive bladder cancer, poorly differentiated stomach cancer, small cell and non-small cell lung cancer, endometrial cancer and prostate cancer (PC). Reduced NEP may promote peptide-mediated proliferation by allowing accumulation of higher peptide concentrations at the cell-surface, and facilitate the development or progression of neoplasia. In PCs, Dr. Nanus Laboratory recently reported that NEP is expressed by androgen-sensitive LNCaP cells and in metastatic PC cells from patients with androgen-dependent disease and NEP expression is diminished in androgen-independent PC cell lines and in the majority (78%) of metastatic androgen-independent PCs *in vivo*. Furthermore, expression of NEP is transcriptionally activated by androgen in LNCaP cells and decreases with androgen-withdrawal. Consequently, we proposed that PC cells which survive androgen-withdrawal will emerge with reduced levels of NEP. This decrease in NEP expression may result in increased growth by allowing PC cells to use neuropeptides as an alternate source to androgen to stimulate cell proliferation. With recombinant NEP and androgen-independent PC cells which expression of cell-surface NEP, we will decipher the mechanisms of NEP induced growth-inhibition (apoptosis, migration, cell cycle arresting, tumorgenicity), expand our understanding of neuropeptides contribution to PC cell growth and survival and explore the impact of NEP on integrin mediated signaling pathways. These studies will lead to a better understanding of the mechanism of NEP's anti-tumor effect in the androgen-independent PC, and provide support for novel approaches for the treatment of advanced prostate cancer.

Body

a. Statement of Works.

The objective of this research proposal is to elucidate and to understand the mechanism of neutral endopeptidase 24.11 (NEP), a cell-surface peptidase which inactivates neuropeptide growth factors at the cell surface, inhibits neuropeptide mediated growth of androgen-independent prostate cancer. The specific aims are:

Task 1. To explore the mechanism by which NEP inhibits prostate cancer cell growth.
    a. characterize the biological effects of NEP androgen-independent PC cells.
    b. establish that recombinant NEP and overexpression of cell-surface NEP induces apoptosis in androgen-independent PC cells.
    c. determine if growth inhibition induced by NEP results from PC cells arresting in a specific phase of the cell cycle.

Task 2. To determine the effect of NEP on integrin mediated signaling pathways
    a to confirm that NEP can inhibit the phosphorylation on tyrosine of focal adhesion kinase (FAK).
    b to assess the affect of NEP on the invasiveness and migration of androgen-independent PC cells.
    c to determine the effect of NEP on neuropeptide-mediated signal transduction.

Task3. To assess the antitumor effects of NEP in an animal model of prostate cancer.
    a. to establish that recombinant NEP can inhibit the tumorigenicity of androgen-independent PC cells in an orthotopic model of PC .
    b. to establish that overexpression of NEP in androgen-independent PC cells inhibits the tumorigenicity of androgen-independent PC cells in an orthotopic model of PC.

b. Studies and Results

During the first year of the fellowship, we have defined the growth inhibitory effects of inducible cell-surface NEP, and further elucidated the mechanism by identifying the novel effect of NEP on signaling. We have also used an orthotopic model of PC to show that overexpression of cell-surface NEP inhibits tumor formation in the prostate.

The mechanism by which NEP inhibits cell growth (Task 1).

To determine the effects on androgen-independent PC cells of overexpressing cell-surface NEP, an inducible tetracycline-regulatory gene expression system was used to stably introduce and express the NEP gene in androgen-independent TSU-Pr1 cells. WT-5 cells expressed high levels of enzymatically active NEP protein when cultured in the absence of tetracycline. TN12 cells, which contain the identical vectors without the NEP gene and do not express NEP, were used as control. Expression of NEP in WT-5 cells following removal of tetracycline from the media resulted in a >80% inhibition in cell proliferation over one week (p<0.005) compared to control cells. Analysis of the mechanisms of NEP-induced growth suppression revealed that NEP expression in WT-5 cells induced a 4-fold increase in the number of PC cells undergoing apoptosis, and increased expression of p21 tumor suprressor

gene protein and in the level of unphosphorylated retinoblastoma protein as determined by Western blot. Flow cytometric analysis show that induced NEP expression in WT-5 cells resulted in a G1 cell cycle arrest. These studies suggest that NEP is a tumor supressive factor in androgen-independent prostate cancer.

The effect of NEP on neuropeptide mediated signaling pathways (Task 2)
To assess NEP's effect on neuropeptide mediated signaling pathways, we used LNCaP cells and hormone-independent, highly invasive TSU-Pr1 PC cells to investigate the mechanisms of NEP action by assessing the effects of NEP on regulating phosphorylation of focal adhesion kinase (FAK) on tyrosine and cell migration. Western analyses and cell migration assays revealed an inverse correlation between NEP expression and the levels of FAK phosphorylation and cell migration in PC cell lines. Constitutively expressed NEP, recombinant NEP and induced NEP expression in WT-5 cells inhibited bombesin and endothelin-1 stimulated FAK phosphorylation and cell migration. These results suggest that NEP plays a crucial role in regulating cell migration which contributes to invasion and metastases in PC cells. We also assay the NEP's effect on neuropeptide mediated AR transactivation. We co-transfected the AR expression vector and the plasmid containing of probasin promoter ARR3 tk-luciferase gene into Swiss 3T3 and PC-3 cells, both of which express high affinity bombesin receptors. Dihydrotestoterone (DHT) increased transcription 7.9 and 17.8 fold at the doses of 0.1and 1.0 nM but had no effect at 10 pM. Bombesin (up to 50 nM) had little effect on luc transcription when added alone. The addition of bombesin to 1 or 10 nM of DHT did not further increase transcription. However, 5-50 nM bombesin + 10 pM DHT resulted in a ~20 fold increase in transcription (p<0.05). This synergistic effect can be blocked by recombinant NEP which hydrolizes bombesin. These data indicate that bombesin can synergize with low (castrate) levels of DHT to induce AR mediated transcription, and suggest that NEP may be involved in AR mediated signaling promoted by neuropeptide in androgen-independent prostate cancer.

To establish that NEP can inhibit the tumorigenicity (Task 3).
We have begun to examine the effects of NEP expression on tumorigenecity in an animal model of PC. We injected 1x10^6 cells (WT5 and N12) orthotopically into the prostate of athymic mice. Tumor formation occurred in the prostate glands of orthotopically injected athymic mice sacrificed at 30 days in 4/5 mice injected with 2 x 10^6 WT-5 cells and fed doxycyline (NEP suppressed), and in all mice injected with TN12 cells fed with or without doxycyline. In contrast, only 1 of 5 mouse prostates developed a tumor in mice injected with WT-5 cells which did not receive doxycylin. We next performed a similar experiment using an orthotopic model of prostate cancer in which PC cells are directly injected into the prostate. Mice were injected and fed with Dox-Diet (doxycycline in the feed) to suppress NEP expression or with regular feed. Tumors only formed in the animals in which NEP expression was suppressed, confirmed by magnetic resonance imaging of the prostate and at autopsy. Ongoing studies are assessing whether induced NEP expression in an established xenograft tumor will inhibit growth and induce tumor regression in vivo.

c. Plans.

The continuing objective of this research proposal is to elucidate and to understand the mechanism of NEP inhibits neuropeptide mediated growth of androgen-independent prostate cancer. In the second year, we will continue our studies aimed at understanding the mechanisms of NEP induced growth inhibition, and the proteins with which NEP interacts to inhibit cell growth. We will continue to determine the involvement of neuropeptide in the androgent receptor mediated transactivation and assess how NEP can regulate this process. In addition, we will continue in vivo studies to show that NEP can inhibit tumor growth in tumor model.

Reportable Outcome

1. Publications

Shen R, Sumitomo M, **Dai J**, Harris A, Kaminetzky D, Gao M, Burnstein KL, Nanus DM. Androgen-induced growth inhibition of androgen receptor expressing androgen-independent prostate cancer cells is mediated by increased levels of neutral endopeptidase. **Endocrinology** 141:1699-74,2000.

Shen R, Sumitomo M, **Dai J**, Hardy DO, Papandreou CN, Shipp M, Freedman LP, Nanus DM. Characterization of two androgen-responsive elements involved in the androgen regulation of the human neutral endopeptidase gene. **Mol Cell Endocrinology** 2000, in press.

Dai J, Shen R, Sumitomo M, Geng Y, Navarro D, Powell CT, Nanus DM. Tumor suppressive effects of neutral endopeptidase in androgen-independent prostate cancer cells. **Clinic Cancer Res,** 2000, submitted.

Sumitomo M, Shen R, Walburg R, **Dai J**, Geng Y, Navarro D, Boileau G, Papandreou CN, Giancotti FG, Knudsen B, Nanus DM. Neutral endopeptidase (CD10, CALLA) inhibits prostate cancer cell migration by blocking neuropeptide-mediated phosphorylation of focal adhesion kinase and by inhibiting phosphoinositide 3-kinase. **J Clin Inv**, 2000, submitted

Dai J, Stahl R, Shen R, Sumitomo M, Navarro D, Bahk J, Gershengorn M, Nanus DM. Bombesin stimulates androgen receptor mediated gene transcription **in preparation**

Abstracts

Dai J, Shen R, Geng, Y, Yang M, Sumitomo M, Powell CT, Garzotto M, Nanus DM. Tumor suppressive effect of neutral endopeptidase in androgen-independent prostate cancer cells. **ProcAACR** 1999:49;638.

Sumitomo M, Shen R, Geng Y, **Dai J**, Navarro D, Knudsen B, Nanus DM. Neutral endopeptidase inhibits cell migration by inhibiting focal adhesion kinase phosphorylation in prostate cancer cells. **ProcAACR** 41:870, 2000

Shen R, Sumitomo M, **Dai J**, Harris A, Burnstein KL, Nanus DM. Increased expression of neutral endopeptidase causes androgen-mediated growth

inhibition of androgen receptor expressing androgen-independent prostate cancer cells. **ProcAACR** 41:429, 2000

Dai J, Stahl R, Shen R, Sumitomo M, Navarro D, Bahk J, Gershengorn M, Nanus DM. Bombesin stimulates androgen receptor mediated gene transcription. **ProcAACR** 41:373, 2000

Conclusions

1. Induction of NEP can induce >80% inhibition in Pc cell proliferation over one week in AIPCs. The mechanisms of NEP-induced growth suppression includes induced a 4-fold increase in the number of PC cells undergoing apoptosis, G1 cell cycle arrest, increased expression of p21 tumor suprressor gene protein and in the level of unphosphorylated retinoblastoma protein.

2. Constitutively expressed NEP, recombinant NEP and induced NEP expression inhibit bombesin and endothelin-1 stimulated FAK phosphorylation and cell migration.

3. Neuropeptide synergies with castrate concentration level of DHT to induce AR mediated transcription. This synergistic effect can be blocked by recombinant NEP.

4. NEP can inhibit the tumorigenicity in an orthotopical animal model.

Androgen-Induced Growth Inhibition of Androgen Receptor Expressing Androgen-Independent Prostate Cancer Cells Is Mediated by Increased Levels of Neutral Endopeptidase*

RUOQIAN SHEN, MAKOTO SUMITOMO, JIE DAI†, ADAM HARRIS‡,
DAVID KAMINETZKY, MIN GAO, KERRY L. BURNSTEIN, AND DAVID M. NANUS

*Urologic Oncology Research Laboratory (R.S., M.S., J.D., A.H., D.K., D.M.N.), Department of Urology,
the Division of Hematology and Medical Oncology (D.M.N.), Department of Medicine, and the
Department of Physiology (M.G.), Joan and Stanford I. Weill Medical College of Cornell University,
New York, New York 10021; and the Department of Molecular & Cellular Pharmacology (K.L.B.),
University of Miami School of Medicine, Miami, Florida 33101*

ABSTRACT

Androgen-mediated growth repression of androgen-independent prostate cancer (AIPC) cells has been reported in androgen-independent PC-3 cells overexpressing the androgen receptor, and in androgen-independent derivatives of LNCaP cells that develop following prolonged culture in androgen-free media. Using two models of AIPC, PC3/AR cells and LNCaP-OM1 cells, a subclone of LNCaP cells derived by prolonged culturing in charcoal-stripped media, we investigated whether expression of neutral endopeptidase 24.11 (NEP), a cell-surface peptidase that cleaves and inactivates neuropeptides implicated in the growth of AIPC, is induced by androgen, and whether NEP contributes to the observed androgen-mediated growth repression. These cell lines each express high levels of androgen receptor. Culturing in dihyrotestosterone (DHT) resulted in a 30–56% (PC3) and 35–43% (LNCaP-OM1) decrease in cell number over 7 days concomitant with a significant increase in NEP enzyme specific activity. Northern analysis detected an increase in NEP transcripts following DHT treatment in PC3/AR cells. The addition of the NEP enzyme inhibitor phosphoramidon to PC3 and LNCaP-OM1 or the NEP competitive inhibitor CGS 24592 to LNCaP-OM1 blocked the increase in NEP enzyme activity and reversed the DHT-induced growth inhibition. Neither phosphoramidon or CGS 24592 alone inhibited cell growth. Furthermore, the reversal of growth inhibition in LNCaP-OM1 cells was dose dependent on the concentration of CGS 24592. These data indicate that androgen-induced growth repression of AIPC cells PC3 and LNCaP-OM1 results in part from androgen-induced expression of NEP in these cells. (*Endocrinology* **141**: 1699–1704, 2000)

THE MOLECULAR events involved in the development of androgen-independent prostate cancer (PC) are not well defined. Potential explanations include increased expression of the bcl-2 proto-oncogene (1), mutations of the p53 tumor suppressor gene (2), increased expression of polypeptide growth factors including epidermal growth factor (EGF), fibroblast growth factors and insulin-like growth factors (IGF) (3), increased expression of neuropeptide growth factors (4, 5), and alterations in the androgen receptor (AR) or AR signaling pathways (6–8). Numerous investigators have examined androgen-independent PC cell lines or androgen-independent sublines of androgen-sensitive LNCaP cells to decipher the mechanisms of androgen-independent growth. PC-3 cells are commonly used as a model for androgen-independent PC. One deficiency of this model is that PC-3

cells express no AR or low levels of nonfunctioning AR (9), in contrast to androgen-independent PC cells *in vivo* in which expression of AR is present and often amplified (7). To study AR function in androgen-independent PCs, researchers have stably introduced a full-length human AR complementary DNA (cDNA) into PC-3 cells (PC3/AR) (10–13). In contrast to LNCaP cells that express a functioning AR and proliferate *in vitro* following treatment with androgen, Yaun *et al.* and Heisler *et al.* each reported a paradoxical inhibition of cell growth in PC3/AR cells cultured in androgen (10, 13). A similar androgen-mediated growth repression has also been reported in androgen-independent derivatives of LNCaP cells that developed following prolonged culture in androgen-free media (14–16). The exact cause of this paradoxical growth inhibition has not been fully explained.

We reported that expression of neutral endopeptidase 24.11 (NEP), a cell-surface peptidase that inactivates neuropeptide growth factors through hydrolysis, is decreased in androgen-independent PC cell lines including PC-3 cells but strongly expressed in androgen-sensitive LNCaP cells (17). Expression of NEP is androgen regulated in PC cells, with expression increasing following DHT treatment and decreasing with androgen withdrawal. Furthermore, overexpression of NEP in androgen-independent PC cells using an inducible vector construct significantly inhibits PC cell

growth (17). We considered whether the DHT-induced growth inhibition observed in PC3/AR cells or in androgen-independent sublines of LNCaP cells resulted from induction of expression of NEP in these cells following treatment with androgen. We report here that androgen-mediated growth repression of these cell types is accompanied by an increase in NEP-specific enzyme activity, and that this growth inhibition can be reversed by cocultivation with an NEP enzyme inhibitor. These data suggest that androgen-induced growth inhibition in AR-expressing, androgen-independent PC cells is mediated in part by NEP.

Materials and Methods

Cell culture

LNCaP cells were maintained in RPMI containing 5% FBS. PC3/AR and PC3/neo cells were cultured in RPMI 1640 without phenol red containing 10% charcoal stripped FBS (HyClone Laboratories, Inc.). LNCaP-OM1 cells are a subclone of LNCaP-OM cells (18) and were maintained in RPMI 1640 without phenol red containing 10% charcoal stripped FBS. NEP enzyme inhibitor phosphoramidon [N-(α-rhamnopyranosyloxy-hydroxyphosphinyl)-leu-trp] and dihydrotestosterone (DHT) were purchased from Sigma (St. Louis, MO). CGS 24592, a competitive inhibitor of NEP, was supplied by Novartis Pharmaceuticals.

Enzyme assays

Cells in logarithmic phase of cell growth were rinsed in cold lysis buffer (50 mM Tris/150 mM NaCl) and lysed in lysis buffer containing 0.5% CHAPS (3-[3-cholamidopropyl-dimethylammonio]-1-propane-sulfonate), which did not affect NEP enzyme specific activity. Protein concentrations were measured using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA). NEP activity was assayed using Suc-Ala-Ala-Ala-Phe-para-aminobenzoate (pAB) (Bachem Bioscience, Inc.) as substrate. Thirty microliters of cell membrane suspension was added to a mixture of 200 μl of 100 mM Tris-HCl, pH.7.6, 10 μl of 20 mM substrate (dissolved in dimethyl sulfoxide), and 10 μl of aminopeptidase N enzyme solution (EC 3.4.11.2; Roche Molecular Biochemicals, Indianapolis, IN), and incubated at 37 C for 10 min. The reaction was stopped by adding 10% trichloroacetic acid, centrifuged at 2500 rpm × 5 min, and 250 μl of supernatant was removed for colorimetric analysis. The absorbance of the chromogen was immediately read at 540 nm against a reaction mixture without cell membrane as blank. Specific activities were expressed as pmol/μg protein/minute and represent an average of at least two separate measurements. The SE of measurement of duplicate experiments was approximately 10 to 20% of the mean value.

Growth assays

Approximately 4,000 cells/well were plated in 12-well tissue culture plates or 10,000 cells/well were plated in six well tissue culture plates (Falcon Division, Becton Dickinson and Co., Cockeysville, MD) in RPMI 1640 10% charcoal-stripped serum for 18 h, counted using a Coulter Counter ZM (Coulter Electronics, Hialeah, FL) (Day 1), and refed with RPMI 1640 10% charcoal-stripped media containing either 30 nM DHT, 10 μM phosphoramidon, or 30 nM DHT plus 10 μM phosphoramidon. DHT was maintained as a 3.4 μM stock dissolved in ethanol, and all cells received an equal concentration of ethanol. Cells were refed on day 3 and counted on day 6. Results represent an average of two independent experiments performed in triplicate. P values were determined using a Student's t test.

Northern analysis

Total RNA was extracted from logarithmically growing cells using RNazol B (Cinna/Biotecx Laboratories, Houston, TX) according to the manufacturer's recommendations. Twenty micrograms of RNA per lane were electrophoresed in 1.2% agarose/formaldehyde gels, transferred to nitrocellulose membranes, and hybridized with a 0.9 kb NEP specific probe fragment containing the 5' end of the NEP cDNA, a PstI/XbaI cut

0.78 kb glyceraldehyde 3 phosphate dehydrogenase (GAPDH) cDNA which were random prime radiolabeled with ^{32}P-dCTP using PrimeIt II (Stratagene Cloning Systems, La Jolla, CA) as per the manufacturer's recommendations.

Protein extraction and Western blot analysis

Protein was extracted from exponentially growing cells and analyzed by Western blotting as previously described (19) using 0.1 μg/ml of anti-AR polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Blots were incubated with enhanced chemiluminescent (ECL) detection reagents (Amersham Pharmacia Biotech, Arlington Heights, IL) and AR protein was detected by autoradiography by exposure of blots to Kodak XAR film for 2–15 min.

Results

Androgen effect on PC3/AR proliferation and NEP enzyme activity

PC3/AR cells contain a stably transfected AR and express AR protein, whereas PC3/neo cells contain the identical vector without the AR and do not express AR protein (12). Northern analysis confirmed that PC3/AR cells express high levels of AR transcripts, whereas PC3/neo cells do not (Fig. 1). We first determined the effects of 30 nM DHT on growth of PC3/AR and PC3/neo cells. In three separate experiments performed in triplicate, PC3/AR cells were 30%, 34% and 56% growth inhibited (P values < 0.001, 0.003 and 0.005, respectively), whereas PC3/neo cells were not significantly inhibited (P value > 0.2 in all three experiments; representative data illustrated in Fig. 2A). To determine if growth inhibition correlated with a change in NEP-specific enzyme activity, PC3/AR and control PC3/neo cells were simultaneously assayed for cell growth and NEP enzyme activity. In all three experiments, NEP enzyme activity increased in cell lysates derived from PC3/AR cultured in DHT but not in lysates from and PC3/neo cells (Table 1, columns Control

PC3/ Neo   **PC3/ AR**
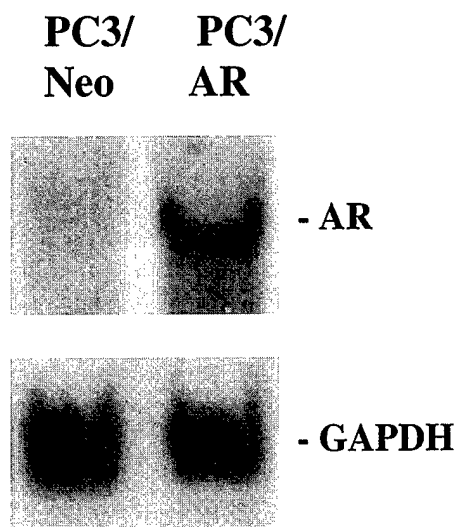

- AR

- GAPDH

FIG. 1. Northern analysis of androgen receptor expression in PC3/AR and PC3/neo cells. Twenty micrograms of RNA extracted from PC3/AR and PC3/neo cells were separated on an agarose gel, transferred to nitrocellulose, and probed with a cDNA probe for the androgen receptor. Note abundant androgen receptor transcripts in PC3/AR cells but not PC3/neo cells. Membrane was stripped and reprobed with a cDNA for GAPDH to confirm equal loading (bottom panel).
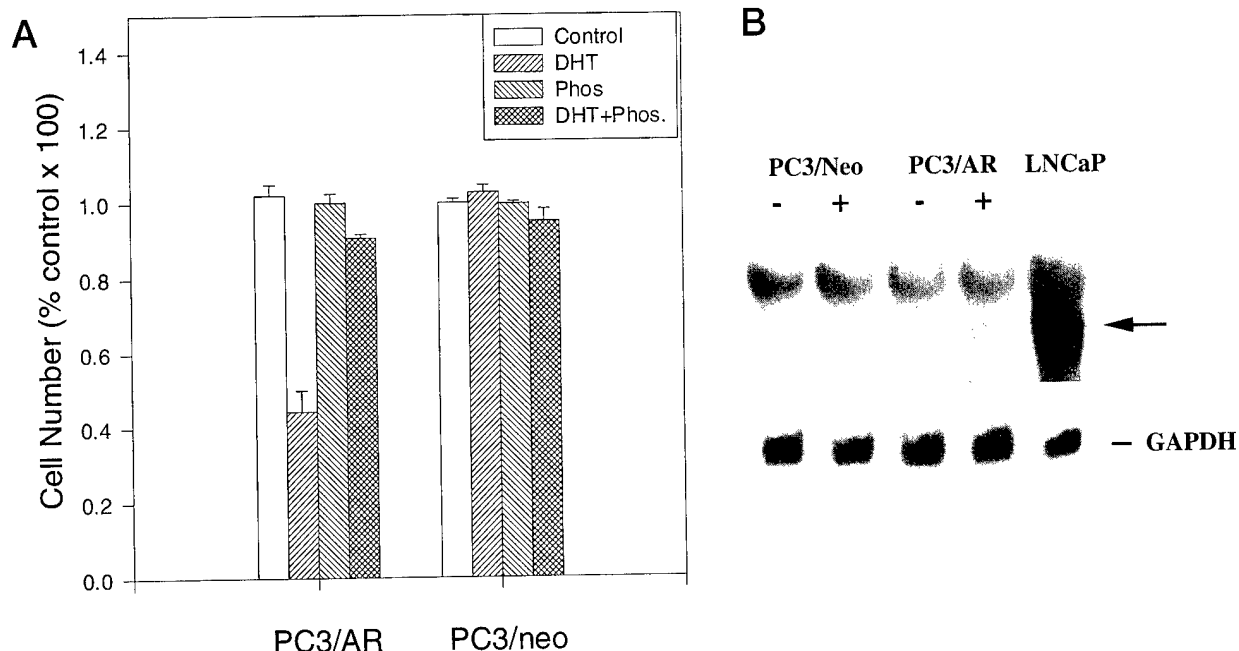
FIG. 2. A, Effect of DHT and phosphoramidon on PC3/AR and PC3/neo cell growth. PC3/AR and PC3/neo cells were seeded in 12-well plates overnight and then refed with RPMI 1640 containing 10% charcoal-stripped serum containing 30 nM DHT. Cell number was determined on Day 6. Data representative of one experiment performed in triplicate on three separate occasions. P value ≤ 0.01 for DHT growth inhibition vs. control, or DHT growth inhibition vs. DHT + phosphoramidon in all three experiments. Enzyme analysis performed simultaneously for all three replicate experiments shown in Table 1. B, Northern analysis of NEP expression in PC3/AR and PC3/neo cells following DHT treatment. RNA was extracted from PC3/AR and PC3/neo cells incubated for 24 h in RPMI containing 10% charcoal stripped serum without (−) or with (+) 30 nm DHT. Twenty micrograms of RNA were separated on an agarose gel, transferred to nitrocellulose, and probed with a cDNA probe for NEP. RNA extracted from LNCaP cells which constitutively express high levels of NEP transcripts was used as control to illustrate NEP transcripts. Note low levels of NEP transcripts in PC3/AR cells treated with DHT in contrast to other cell lines. Membrane was stripped and reprobed with a cDNA for GAPDH to confirm equal loading (bottom panel). Arrow, NEP transcript. Band above arrow in all lanes represents cross-hybridization of the NEP probe to 28s ribosomal RNA.

TABLE 1. NEP enzymatic activities for PC3/AR and PC3/neo incubated in 30 nM DHT

	Control[a]	DHT	Phosphoramidon	DHT + Phosphoramidon
PC3/AR	6.5 (0.15)	14.6 (1.3)	6.6 (0.18)	6.4 (0.13)
PC3/neo	6.2 (0.09)	6.3 (0.19)	6.0 (0.12)	6.2 (0.12)

PC3/AR and PC3/Neo cells were grown in media containing 10% charcoal-stripped serum (control), 30 nM DHT, 10 μM phosphoramidon or both DHT + phosphoramidon. NEP-specific enzyme activity increased from approximately 6 to 15 pmol/μg protein/min, which was completely blocked by phosphoramidon. Enzyme activity of PC3/Neo cells did not change. Data shown average of three separate experiments. P value < 0.005 for three separate experiments comparing DHT effect on PC3/AR cells to all other conditions. Growth assays were performed in triplicate simultaneously for each experiment. Representative data shown in Fig. 2A.
[a] All values expressed as pmol/μg protein/min. ( ) indicate SE.

and DHT). To determine if growth inhibition resulted from an increase in NEP enzyme activity, 10 μM of the NEP enzyme inhibitor phosphoramidon (20) was added to the media on day 3 of a 6-day growth assay. Phosphoramidon alone did not significantly affect growth of PC3/AR or PC3/neo cells. Measurement of NEP enzyme activity confirmed that phosphoramidon blocked the increase in enzyme activity observed following incubation with DHT in PC3/AR cells (Table 1). Analysis of cell number indicated that incubation with DHT and phosphoramidon resulted in complete abrogation of the DHT-induced growth inhibition observed in

PC3/AR cells (Fig. 2A). These data suggest that DHT-induced growth inhibition in PC3/AR cells results from an increase in NEP enzyme activity.

DHT induces NEP transcripts in PC3/AR cells

LNCaP cells constitutively express NEP transcripts (Fig. 2B, lane 5), while PC3/AR and PC3/neo cells grown in charcoal-stripped media do not express detectable NEP transcripts by Northern analysis (Fig. 2B, lanes 1 and 3). However, incubation of PC3/AR and PC3/neo cells in 30 nM of DHT for 24 h resulted in the expression of low levels of detectable NEP transcripts in PC3/AR cells but not PC3/neo cells (Fig. 2B, lanes 2 and 4).

DHT inhibits growth of LNCaP-OM1 cells and induces NEP enzyme activity

Our data on PC3/AR cells suggested that DHT-mediated expression of NEP resulted in growth inhibition. We next examined a second model of AR expressing androgen-independent PC in which DHT-mediated growth repression had previously been reported. LNCaP-OM cells were originally derived by culturing parental LNCaP cells in media containing charcoal stripped serum for over 12 months (18). LNCaP-OM1 cells, which express high levels of AR protein (Fig. 3A), are a subline obtained by dilutional cloning of LNCaP-OM cells.
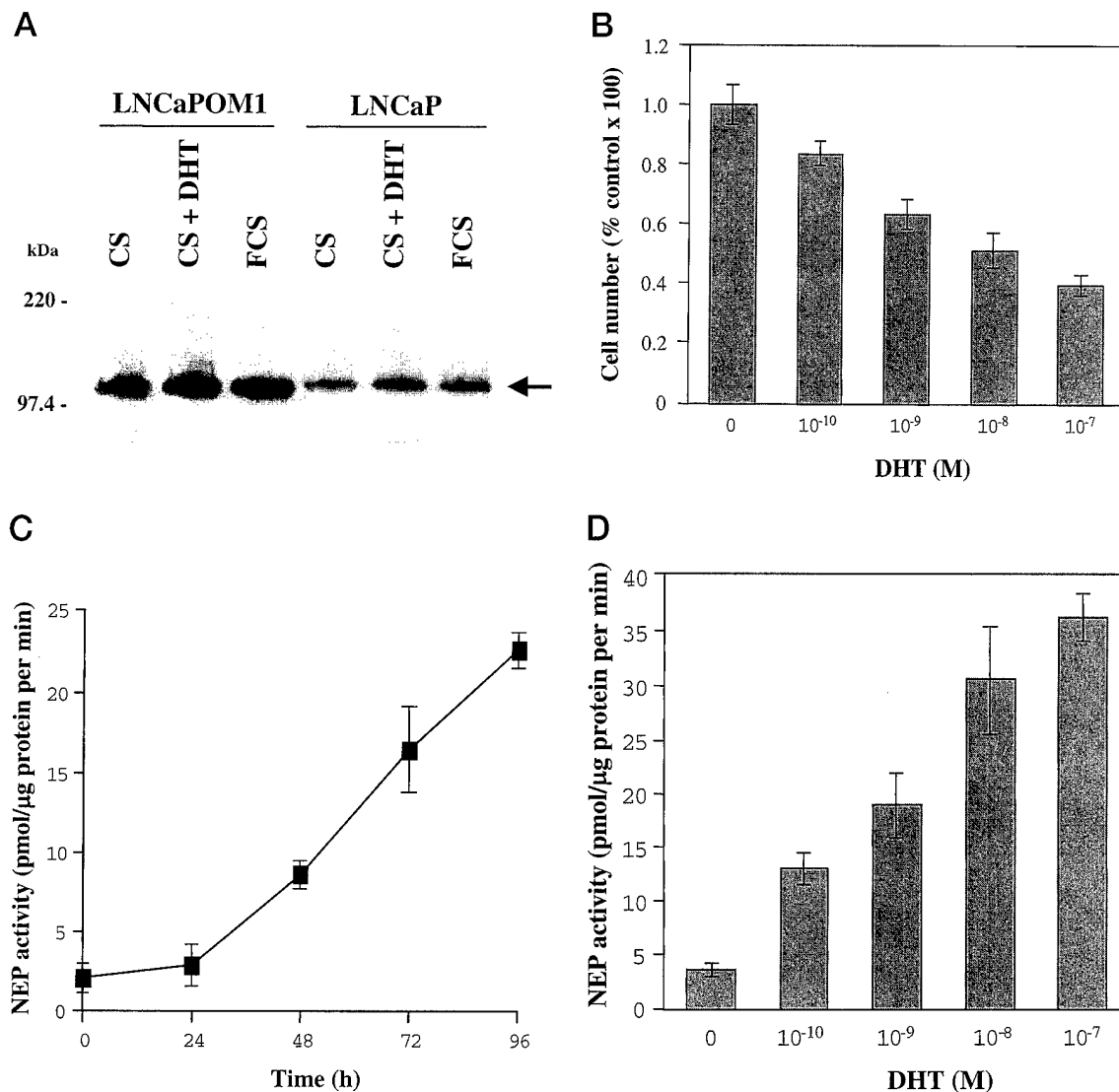
FIG. 3. A, Western Analysis of AR expression in LNCaP-OM1 and LNCaP cells. Cell lysates derived from LNCaP-OM1 and LNCaP cells grown in media containing charcoal-stripped serum (CS), charcoal strip serum plus 30 nM DHT (CS plus DHT), or FCS were separated on SDS-DAGE gel transferred to nitrocellulose and immunoblotted with a monoclonal antibody that recognizes AR. Note the high levels of AR that are expressed by both cell lines under all three conditions. B, Effect of increasing concentrations of DHT on growth of LNCaP-OM1 cells. LNCaP-OM1 cells were seeded in 12-well plates overnight and refed with RPMI containing 10% charcoal strip serum with increasing concentrations of DHT. Cell number was determined on day 6. All experiments were performed in triplicate on three separate occasions. The data are representative of one experiment. C and D, Effect of DHT on NEP Specific enzyme activity in LNCaP-OM1 cells. LNCaP-OM1 cells were cultured RPMI containing 10% charcoal-stripped serum with the addition of DHT. C, Cell cultured in 1 nM DHT and NEP-specific enzyme activity measured every 24 h for 4 days. Note increase in enzyme activity over time. D, Enzyme activity measured with increasing concentrations of DHT following 4-day incubation.

Incubation of LNCaP-OM1 cells in media containing DHT for 7 days resulted in a dose-dependent decrease in cell number (Fig. 3B). This was accompanied by a significant increase in NEP specific enzyme activity measured over 96 h (Fig. 3C). Furthermore, the increase in NEP enzyme activity was dependent on the concentration of DHT (Fig. 3D). These results were similar to those observed with PC3/AR cells.

NEP inhibitors reverse DHT-induced growth inhibition of LNCaP-OM1 cells

As illustrated in Fig. 4A, the addition of 10 μM phosphoramidon to the media reversed the DHT-induced growth

inhibition of LNCaP-OM1 cells, suggesting that growth inhibition results from an increase in NEP enzyme activity. This was accompanied by a significant decrease in NEP enzyme activity (data not shown). Phosphoramidon can inhibit other peptidases in addition to NEP. Therefore, to confirm that NEP inhibition reversed DHT induced growth inhibition in LNCaP-OM1 cells, we obtained the NEP competitive inhibitor CGS 24592 (21). While incubation in media containing 10 nM CGS 24592 had no effect on cell growth, 10 nM of CGS 24592 reversed the growth inhibition resulting from 1 nM DHT (Fig. 4B). NEP specific enzyme activity in LNCaP-OM1 cells grown in 1 nM DHT decreased from 27.7 pmol/μg

A.

Cell number (% control x 100)

1.2
1.0
0.8
0.6
0.4
0.2
0

Con   + DHT   + Phosp   + DHT + Phosp

B.

Cell number (% control x 100)

1.2
1.0
0.8
0.6
0.4
0.2
0

Con   + DHT   + CGS   + DHT + CGS

C.

Cell number (% control x 100)

1.2
1.0
0.8
0.6
0.4
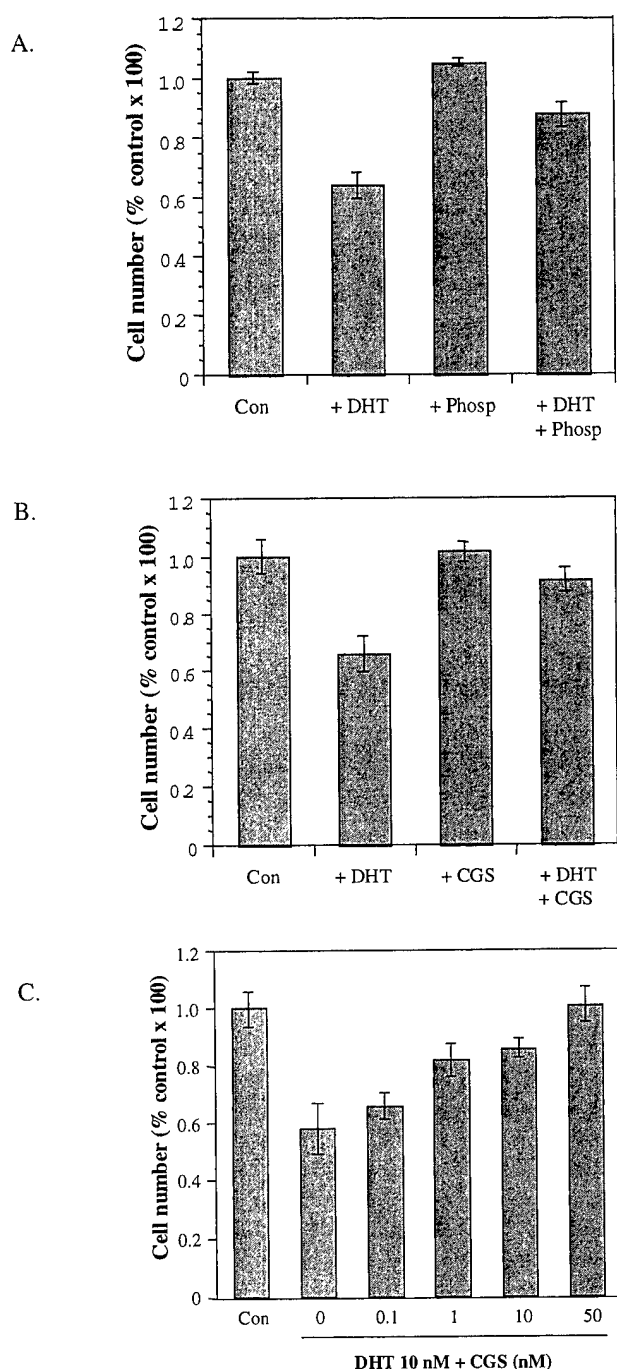0.2
0

Con   0   0.1   1   10   50

DHT 10 nM + CGS (nM)

FIG. 4. Effect of NEP inhibitors phosporamidon and CGS 24592 on LNCaP-OM1 cell growth. A, LNCaP-OM1 cells were seeded in 12-well plates overnight and then refed with RPMI 1640 containing 10% charcoal-stripped serum (Control), charcoal-stripped serum plus 1 nM DHT, charcoal-stripped serum plus 10 μM phosphoramidon (Phosp), or both DHT and phosporamidon. Note that growth inhibition induced by DHT is reversed by the addition of phosporamidon ($P < 0.005$), whereas phosphoramidon alone has no effect on cell growth. Data representative of one experiment performed in triplicate on three separate occasions. B, Identical experiment as panel A except 10 nM CGS 24592 used instead of phosphoramidon ($P < 0.005$). C, LNCaP-OM1 cells were cultured RPMI containing 10% charcoal strip serum with the addition of 10 nM DHT and increasing concentrations of CGS 24592. Note reversal of DHT growth inhibition is more pronounced with higher concentrations of CGS 24592.

protein/min to 0.3 pmol/μg protein/min (P value < 0.005) following incubation with 10 nM CGS 24952 concomitant with the reversal of DHT-induced growth inhibition. This reversal of DHT-induced growth inhibition was dose dependent on the concentration of CGS 24592 (Fig. 4C).

Discussion

The objective of this study was to determine if an increase in NEP expression and associated catalytic activity could account for the paradoxical growth inhibition observed following incubation in androgen in PC-3 cells expressing AR and in an androgen-independent derivative of LNCaP cells. PC3/AR cells were generated to better delineate the role of the AR in the development and progression of hormone-refractory PC. Androgen may increase the proliferation of these cells because androgen is a growth and survival factor for PC cells. However, as previously reported in PC3/AR cells (12, 13), the addition of androgen results in significant growth inhibition. Our data implicate an androgen-induced increase in NEP enzyme activity as a cause for androgen-induced growth inhibition in PC3/AR cells. Similar results implicating androgen-induced expression of NEP as a contributing factor to growth inhibition were obtained with a second androgen-independent, AR expressing PC cell line derivative of LNCaP cells, LNCaP-OM1. In both of these cell lines, androgen-induced growth inhibition was reversed with the NEP enzyme inhibitor phosphoramidon. Furthermore, a similar reversal of growth inhibition was observed in LNCaP-OM1 cells using a second, more specific NEP inhibitor CGS 24592. The effects of CGS 24592 were dose dependent as would be expected with a competitive enzyme inhibitor. Of interest, parental LNCaP cells exhibit a biphasic growth response to androgens, with growth stimulation at lower, physiologic concentrations of androgens and a reduction in growth stimulation as the concentration of androgen is increased in the media (22). The etiology of this response to androgen in LNCaP cells is unknown, but our preliminary data indicate NEP inhibitors do not reverse the reduction in LNCaP growth at high androgen concentrations, suggesting this phenomenon may not be mediated by NEP.

The increase in NEP expression following androgen treatment results from the fact that the NEP gene is transcriptionally activated by androgen (17). Progesterone also increases NEP messenger RNA and protein expression in human endometrial stromal cells (23), and glucocorticoids increase NEP expression in human bronchial epithelial BEAS-2B cells (24) and in human vascular smooth muscle cells (25), indicating that the NEP gene is regulated by this family of steroid hormones. We have identified an androgen response element (ARE) located in exon 24 of the NEP gene and a second androgen response region located in the NEP promoter that bind AR and activate transcription of a reporter gene in response to androgen treatment (26). Thus, the introduction of DHT into the media of these cells stimulates NEP transcription. The less pronounced increase in NEP activity in PC-3 cells compared with LNCaP-OM1 may result from the fact that the NEP promoter contains a 5' CpG island spanning the transcriptional regulatory region (27, 28), and this region that contains the androgen response region is hypermethylated in PC-3 cells (29). Therefore, transcriptional activation of the NEP gene in response to steroid induction may be limited.

The mechanism of NEP growth inhibition in PC cells involves hydrolysis of neuropeptides such as bombesin and endothelin-1, or other unknown peptides important in androgen-independent growth (30, 31). PC-3 and LNCaP cells each express cell-surface receptors for the NEP substrates bombesin (32) and endothelin-1 (29, 33). An increase in NEP expression in PC3/AR or LNCaP-OM1 cells following DHT treatment would inactivate these neuropeptides. Sudden loss of these neuropeptides may have an immediate adverse effect on cell proliferation. The resulting growth inhibition may be moderate because neuropeptides are not strong mitogens but appear to interact with other polypeptide growth factors such as epidermal growth factor and insulin-like growth factors to stimulate cell growth (5, 33).

Other explanations have been implicated as a cause for androgen-mediated growth repression of androgen-independent PC cells. These include increased number of cells in G1 and an increase in the number of cells undergoing apoptosis in DHT treatment of PC3/AR cells (13) and induced expression of p27Kip1 in another androgen-independent LNCaP subline 104-R1 (15). Our results are compatible with these explanations because overexpression of NEP can result in growth arrest, apoptosis and altered expression of cyclin-dependent kinases (unpublished data).

In conclusion, these experiments further implicate NEP in the development and progression of androgen-independent PC. NEP is normally expressed by prostate epithelial cells, *in vitro* by androgen-sensitive LNCaP cells and *in vivo* in metastatic PC cells from patients with androgen-dependent disease. NEP expression is diminished in androgen-independent PC cell lines and in the majority of metastatic androgen-independent PCs *in vivo*. Overexpression of NEP in another androgen-independent PC cell line, Tsu-Pr1 cells, inhibits growth (17). Similarly, induction of NEP expression in PC3/AR and LNCaP-OM1 cells inhibits growth. Taken together, these independent experiments firmly establish a link between increased NEP activity and inhibition of androgen-independent PC cellular proliferation.

Acknowledgments

References

1. McDonnell TJ, Troncoso P, Brisbay SM, Logothetis C, Chung LW, Hsieh JT, Tu SM, Campbell ML 1992 Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. Cancer Res 52:6940–6944
2. Bookstein R, MacGrogan D, Hilsenbeck SG, Sharkey F, Allred DC 1993 p53 is mutated in a subset of advanced-stage prostate cancers. Cancer Res 53:3369–3373
3. Russell PA, Bennett S, Stricker P 1998 Growth factor involvement in progression of prostate cancer. Clin Chem 44:705–723
4. Di Sant'Agnese PA 1995 Neuroendocrine differentiation in prostatic carcinoma. Cancer 75:1850–1859
5. Aprikian AG, Han K, Guy L, Landry F, Begin LR, Chevalier S 1998 Neuroendocrine differentiation and the bombesib/gastrin releasing peptide family of neuropeptides in the progresssion of human prostate cancer. Prostate 8:52–61
6. Taplin ME, Bubley GJ, Shuster TD, Frantz ME, Spooner AE, Ogata GK, Keer HN, Balk SP 1995 Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. N Engl J Med 332:1393–1398
7. Koivisto P, Kononen J, Palmberg C, Tammela T, Hyytinen E, Isola J, Trapman J, Cleutjens K, Noordzij A, Visakorpi T, Kallioniemi OP 1997 Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. Cancer Res 57:314–319
8. Miyamoto H, Yeh S, Wilding G, Chang C 1998 Promotion of agonist activity

9. Tilley WD, Bentel JM, Aspinall JO, Hall RE, Horsfall DJ 1995 Evidence for a novel mechanism of androgen resistance in the human prostate cell line, PC-3. Steroids 60:180–186
10. Yuan S, Trachtenberg J, Mills GB, Brown TJ, Xu F, Keating A 1993 Androgen-induced inhibition of cell proliferation in an androgen- insensitive prostate cancer cell line (PC-3) transfected with a human androgen receptor complementary DNA [published erratum appears in Cancer Res 1995, 55:719]. Cancer Res 53:1304–1311
11. Brass AL, Barnard J, Patai BL, Salvi D, Rukstalis DB 1995 Androgen up-regulates epidermal growth factor receptor expression and binding affinity in PC3 cell lines expressing the human androgen receptor. Cancer Res 55:3197–3203
12. Dai JL, Maiorino CA, Gkonos PJ, Burnstein KL 1996 Androgenic up-regulation of androgen receptor cDNA expression in androgen-independent prostate cancer cells. Steroids 61:531–539
13. Heisler LE, Evangelou A, Lew AM, Trachtenberg J, Elsholtz HP, Brown TJ 1997 Androgen-dependent cell cycle arrest and apoptotic death in PC-3 prostatic cell cultures expressing a full-length human androgen receptor. Mol Cell Endocrinol 126:59–73
14. Umekita Y, Hiipakka RA, Kokontis JM, Liao S 1996 Human prostate tumor growth in athymic mice: inhibition by androgens and stimulation by finasteride. Proc Natl Acad Sci USA 93:11802–11807
15. Kokontis JM, Hay N, Liao S 1998 Progression of LNCaP prostate tumor cells during androgen deprivation: hormone-independent growth, repression of proliferation by androgen, and role for p27Kip1 in androgen-induced cell cycle arrest. Mol Endocrinol 12:941–953
16. Gao M, Ossowski L, Ferrari AC 1999 Activation of Rb and decline in androgen receptor protein precede retinoic acid-induced apoptosis in androgen-dependent LNCaP cells and their androgen-independent derivative. J Cell Physiol 179:336–346
17. Papandreou CN, Usmani B, Geng YP, Bogenrieder T, Freeman RH, Wilk S, Finstad CL, Reuter VE, Powell CT, Scheinberg D, Magill C, Scher HI, Albino AP, Nanus DM 1998 Neutral endopeptidase 24.11 loss in metastatic human prostate cancer contributes to androgen-independent progression. Nature Med 4:50–57
18. Platica M, Verma RS, Macera MJ, Platica O 1997 LNCaP-OM, a new androgen-resistant prostate cancer subline. In Vitro Cell Dev Biol 33:147–149
19. Papandreou CN, Bogenrieder T, Loganzo F, Chao MV, Nanus DM, Albino AP 1996 Mutation and expression of the low affinity neurotropin receptor in human malignant melanoma. Melanoma Res 6:373–378
20. Fournie-Zaluski MC, Perdrisot R, Gacel G, Swerts JP, Roques BP, Schwartz JC 1979 Inhibitory potency of various peptides on enkephalinase activity from mouse striatum. Biochem Biophys Res Commun 91:130–135
21. Trapani AJ, Smits JFM, Sun XJ, Webb RL, Yau ET 1994 CGS 24128: a long-acting inhibitor of neutral endopeptidase 3.4.24.11. Cardiovasc Drug Rev 12:271–285
22. Joly-Pharaboz MO, Soave MC, Nicolas B, Mebarki F, Renaud M, Foury O, Morel Y, Andre JG 1995 Androgens inhibit the proliferation of a variant of the human prostate cancer cell line LNCaP. J Steroid Biochem Mol Biol 55:67–76
23. Casey ML, Smith JW, Nagai K, Hersh LB, MacDonald PC 1991 Progesterone-regulated cyclic modulation of membrane metalloendopeptidase (enkephalinase) in human endometrium. J Biol Chem 34:23041–23047
24. van der Velden VH, Naber BA, van der Spoel P, Hoogsteden HC, Versnel MA 1998 Cytokines and glucocorticoids modulate human bronchial epithelial cell peptidases. Cytokine 10:55–65
25. Graf K, Schaper C, Grafe M, Fleck E, Kunkel G 1998 Glucocorticoids and protein kinase C regulate neutral endopeptidase 24.11 in human vascular smooth muscle cells. Basic Res Cardiol 93:11–17
26. Shen R, Dai J, Sumitomo M, Hardy DO, Usmani B, Papandreou C, Hersh LB, Shipp MA, Freedman LP, Nanus DM, Identification and characterization of two androgen response regions in the human neutral endopeptidase gene. Mol Cell Endocrinol, in press
27. D'Adamio L, Shipp MA, Masteller EL, Reinherz EL 1989 Organization of the gene encoding common acute lymphoblastic leukemia antigen (neutral endopeptidase 24.11): multiple miniexons and separate 5′ untranslated regions. Proc Natl Acad Sci USA 86:7103–7107
28. Ishimaru F, Shipp MA 1995 Analysis of the human CD10/neutral endopeptidase 24.11 promotor region: two separate regulatory elements. Blood 85:3199–3207
29. Usmani B, Shen R, Janeczko M, Papandreou CN, Lee W-H, Nelson WG, Nelson JB, Nanus DM, Methylation of the neutral endopeptidase gene CpG island in human prostate cancer. Clin Cancer Res, in press
30. Kenny AJ, O'Hare MJ, Gusterson BA 1989 Cell-surface peptidases as modulators of growth and differentiation. Lancet 2:785–787
31. Shipp MA, Look AT 1993 Hematopoietic differentiation antigens that are membrane-associated enzymes: cutting is the key! Blood 84:1052–1070
32. Reile H, Armatis PE, Schally AV 1994 Characterization of high-affinity receptors for bombesin/gastrin releasing peptide on the human prostate cancer cell lines PC-3 DU-145: internalization of receptor bound 125(supercript 125)I-(Tyr supercript 4) bombesin by tumor cells. Prostate 25:29–38
33. Nelson JB, Chantack K, Hedican SP, Magnuson SR, Opgenorth TJ, Bova GS, Simons JW 1996 Endothelin-1 production and decreased endothelin B receptor expression in advanced prostate cancer. Cancer Res 56:663–668

Identification and characterization of two androgen response regions in the human neutral endopeptidase gene

Ruoqian Shen, Makoto Sumitomo, Jie Dai, Dianne O. Hardy, Daniel Arroyo, Badar Usmani, Christos N. Papandreou, Louis B. Hersh, Margaret A. Shipp, Leonard P. Freedman, David M. Nanus *

The New York Presbyterian Hospital, 520 E. 70th Street, ST-341, New York, NY 10021, USA

Abstract

Transcription of the human neutral endopeptidase 24.11 (NEP) gene is androgen regulated in prostate cancer cells. Homology search identified a sequence GTCACAaagAGTTCT similar to the ARE consensus sequence GGTACAnnnTGTTCT within the 3′-untranslated region of the NEP mRNA. A double-stranded radiolabelled oligonucleotide containing this NEP-ARE sequence formed a DNA-protein complex with nuclear proteins from LNCaP cells or CV-1 cells co-transfected with an androgen receptor (AR) expression vector, and with full-length AR synthesized by baculovirus in mobility shift assays. Unlabeled NEP-ARE or consensus ARE but not mutated NEP-ARE replaced radiolabelled NEP-ARE. Steroid-dependent enhancement of transcription was assayed by transfecting ptkCAT reporter constructs containing the NEP-ARE into CV-1/AR cells and prostate cancer cells (PC-3/AR). Enhancement of chloramphenicol acetyltransferase (CAT) activity was increased four-fold by androgen, seven-fold by dexamethasone and three-fold by progesterone in CV-1/AR cells, and the NEP-ARE bound to glucocorticoid and progesterone receptor in mobility shift assays. We next performed DNase-I footprinting analysis of the NEP promoter and identified a 23 bp sequence GGTGCGGGTCGGAGGGATGCCCA (NEP-ARR) which was protected from DNase I cleavage by nuclear extracts from COS-7 cells expressing AR. This sequence was 62.5% homologous to an androgen responsive region (PSA-ARR) identified in the promoter of the prostate specific antigen (PSA) gene. A double-stranded radiolabelled oligonucleotide containing this NEP-ARR sequence formed DNA-protein complex with AR but not GR proteins. Unlabeled NEP-ARR, PSA-ARR and NEP-ARE replaced radiolabelled NEP-ARR. Steroid-dependent enhancement of transcription assays in PC-3/AR cells revealed that the enhancement of CAT activity was increased 2.3-fold by androgen, but not by glucocorticoid or progesterone. In a thymidine kinase promoter, the NEP-ARE and NEP-ARR together stimulated a five-fold increase in promoter activity in PC cells. These data suggest that steroid regulation of the NEP gene involves at least two elements including a typical ARE which binds androgen, progesterone and glucocorticoid receptors, and a unique ARR which only binds androgen. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Androgen regulation; Androgen response element; Neutral endopeptidase; Prostate cancer

1. Introduction

Neutral endopeptidase 24.11 (NEP, neprilysin, enkephalinase, CD10, EC 3.4.24.11) is a 90–110 kD zinc dependent cell-surface metallopeptidase expressed by prostatic epithelial cells which cleaves peptide bonds on the amino side of hydrophobic amino acids. NEP inactivates a variety of physiologically active neuropeptides which have been implicated in prostate cancer progression, including neurotensin, bombesin and endothelin-1 (Aprikian et al., 1998). We reported that NEP expression is decreased in androgen-independent PC cell lines and in the majority (78%) of metastatic androgen-independent PCs in vivo (Papandreou et al., 1998). In addition, we showed that expression of the NEP gene is transcriptionally activated by androgen and decreases with androgen-withdrawal in androgen-sensitive LNCaP cells (Papandreou et al., 1998). These

* Corresponding author. Tel.: +1-212-7462920; fax: +1-212-7466645.

E-mail address: dnanus@mail.med.cornell.edu (D.M. Nanus).

data are consistent with a model in which androgen-withdrawal allows neuropeptide-mediated actions by downregulating NEP and facilitating the development of a neuropeptide-stimulated androgen-independent PC cell population.

The human NEP gene spans more than 80 kb and is composed of 24 exons. Exons 1 and 2 encode 5'-untranslated sequences; exon 3 encodes the initiation codon and includes the cytoplasmic and transmembrane domains (D'Adamio et al., 1989). Twenty short exons (exons 4–23) ranging in size from 36 to 162 bp encode most of the extracellular portion of the enzyme. Exon 24 encodes the COOH-terminal 32 amino acids of the protein and contains the entire 3'-untranslated region (UTR) (D'Adamio et al., 1989). Two separate regulatory regions control the transcription of 5' alternatively spliced NEP transcript. These type 1 and type 2 NEP regulatory regions are both characterized by the presence of multiple transcription initiation sites, the absence of classic TATA boxes and consensus initiator elements, and are believed to control tissue-specific expression (Li et al., 1995). In the majority of tissues examined to date, type 2 NEP transcripts were more abundant while the amount of type 1 transcripts was more variable with the highest type 1 levels in fetal thymus and lymphoblastic leukemia cell lines (Ishimaru and Shipp, 1995). A previous analysis of the major type 2 promoter identified three functionally active transcription factor binding sites in the human acute lymphoblastic leukemia cell line Nalm-6 and in SV40-transfromed human fetal bronchial epithelial FHTE56 cells (Ishimaru et al., 1997). Region I (bp $-145/-116$) contains an inverted CCAAT sequence which binds the transcription factor CBF/NF-YA isoforms, while region II (bp $-93/-53$) binds a putative positive regulatory element and region III (bp $-53/-23$) binds a putative negative regulatory element (Ishimaru et al., 1997).

The mechanisms underlying the regulation of NEP gene by androgen in prostate cancer cells are unknown. Progesterone increases NEP mRNA and protein expression in human endometrial stromal cells (Casey et al., 1991), and glucocorticoids increase NEP expression in SV40-transformed human tracheal epithelial cells (Borson and Gruenert, 1991), human bronchial epithelial BEAS-2B cells (van der Velden et al., 1998), and in human vascular smooth muscle cells (Graf et al., 1998). These data suggest the presence of hormone response elements (HRE) in the NEP DNA that can bind androgen (AR), glucocorticoid (GR) and progesterone receptors (PR) and regulate transcription. The HRE for this subfamily consists of two imperfect 6-base pair indirect repeats separated by a spacing of 3 nucleotides, GGA/TACAnnnTGTTCT (Roche et al., 1992; Rundlett and Miesfeld, 1995). This sequence mediates androgen-induced, as well as glucocorticoid- and progesterone-in-

duced gene expression. Steroid inducibility is limited by tissue-specific expression of steroid hormone receptors and protein-protein interactions with other transcription factors (Mader et al., 1993). Many androgen responsive elements (AREs) consist of HRE-like sequence with an inverted binding site separated by a 3-bp nucleotide spacer. Other AREs contain only a single binding site sequence (Rennie et al., 1993; Cleutjens et al., 1996). To characterize the steroid hormone regulation of the NEP gene, we focused on the identification of the cis-elements involved in androgen regulation. We report the identification and characterization of a typical inverted repeat sequence ARE located in exon 24 (NEP-ARE) of the NEP gene which binds AR, GR and PR. In addition, we report the identification and characterization of an androgen response region (ARR) located in the NEP promoter (NEP-ARR) which preferentially binds AR. These two elements interact to activate transcription of a reporter gene in response to the androgen treatment.

2. Experimental procedures

2.1. Cell lines and tissue culture

PC-3/AR (PC-3 cells expressing human AR after stable transfection of AR cDNA kindly supplied by Dr K. Burnstein (Dai et al., 1996), LNCaP, CV-1 and COS-7 cells were maintained in RPMI 1640 or MEM containing 10% fetal bovine serum (FBS). Experiments using dihydrotestosterone (DHT), dexamethasone or progesterone were performed in RPMI1640 or MEM without phenol red containing 10% charcoal stripped fetal calf serum (FCS) (Hyclone Laboratories).

2.2. Nuclear extract preparation

Nuclear extracts were prepared as described (Freedman and Alroy, 1993). Briefly, monolayer cells were collected and lysed in hypotonic buffer A [10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)], followed by extraction in cold buffer C [20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.2 mM PMSF]. After centrifugation at 20 000 × g, the supernatants were dialyzed against buffer D [100 mM KCl, 20 mM HEPES (pH 7.9), 0.1% NP-40, 10% gycerol, 1 mM DTT, 1 mM EDTA] and stored at $-80°C$.

2.3. Gel mobility shift assays

Binding reactions were carried out in 20 μl binding buffer [0.5 mM HEPES-KOH, pH 7.9, 50 mM KCl, 1 mM DTT, 1 mM EDTA and 5% (v/v) glycerol], 2 μg of

poly[dI-dC] (polydeoxyinosinic-deoxycytidylic acid), and 0.3–0.5 ng (2–3 × 10³ cpm) of end-labeled oligonucleotide. Nuclear extracts (2–4 μg protein) or purified AR, GR or PR proteins (60–100 ng) were added, and the mixture was incubated at room temperature for 20 min, and terminated by the addition of 2 μl of loading buffer [6.7 mM Tris–HCl, pH 7.9, 3.3 mM NaOAc, 50% (v/v) glycerol, 0.25% (w/v) bromophenol blue, and 0.25% (w/v) xylene cyanol]. The DNA/protein complexes were then resolved on non-denaturing polyacrylamide gels with the ion strength of 0.375 × TBE (Tris–borate-EDTA buffer, pH 8.0). Androgen receptor (AR) was produced in baculovirus and prepared as described (Vegeto et al., 1992). Human progesterone receptor B form was made by coupled in vitro transcription/translation using the Promega TNT Reticulocyte Lysate and was a gift from Milan Bagchi of the Population Council's Center for Biomedical research. Purified glucocorticoid receptor (GR) (DNA binding domain, DBD) was a kind gift from Dr K. Yamamoto (University of California, San Francisco, CA). Oligonucleotides containing the sequence of the ARE derived from the rat prostate C3 gene were used in competition assays (Sense 5′-GTTTGAACATAGTACGTCAT-GTTCTCAAGATAG-3′) (Tan et al., 1992).

2.4. Construction of various recombinant DNAs containing NEP-ARE and NEP-ARR

Oligonucleotides NEP-ARE: sense 5′-AGCTTA-TAAGTCACAAAGAGTTCTGGAAAAT-3′ and antisense 5′-CTAGATTTTCCAGAACTCTTTGTGACT-TATA-3′; mutated NEP-ARE; sense 5′-AGCTTA-TAAGTC GTAAAGA CATTTGGAAAAT-3′ and antisense (5′-CTAGATTTTCCA AATGTCTTT ACGA-CTTATA-3′ (mutated bases are underlined) were phosphorylated by T4 kinase reaction with rATP, and ligated to form multi-oligonucleotides, which were then ligated into the HindIII site upstream of the tk promoter in the ptkCAT plasmid [thymidine kinase promoter and chloramphenicol acetyltransferase (CAT) gene]. The constructs are called: p3NAREtkCAT, three copies of NEPARE; p1NAREtkCAT, one copy of NE-PARE; pNARE(M)tkCAT, one copy of mutated NE-PARE. The copy number of inserts was checked by restriction digestion and resolution on a polyacrylamide gel, and further confirmed by sequencing. Complementary DNAs derived from mRNA prepared from LN-CaP cells were amplified as described (Papandreou et al., 1998) using the oligonucleotide primers (sense; 5′-A AAAAGCTTGTGAACTCATTGCTCCCTAAGA-CT-3′, antisense: 5′-CAATAGGATCCGAAAAGA-GAGGAACAGAAGC-3′) to generate a 292 bp RT-PCR product containing 203 bp 5′- and 74 bp 3′-flanking regions around the core NEP-ARE sequence.

The amplified product was digested with HindIII and BamHI, then inserted upstream of the tk promoter to construct pNARE(L)tkCAT. DNA sequencing of plasmids was performed on a Perkin–Elmer Biosystems 377 sequencer using AmpliTaq®DNA Polymerase (Perkin–Elmer Inc.).

The plasmids containing NEP-ARRs were constructed using a similar strategy as that of p3AREtkCAT. Oligonucleotides of NEP-ARR possess the sequences: sense 5′-AGCTTTGGGTGCGGGTCG-GAGGGATGCCCAGGTGCG-3′, antisense 5′-GATCCGCACCTGGGCATCCCTCCGACCCGCA-CCCAA-3′; PSA-ARR: sense 5′-AGCTTGTGGT-GCAGGGATCAGGGAGTCTCACAATCG-3′, antisense 5′-GATCCGATTGTGAGACTCCCTGATCC-CTGCACCACA-3′. Self-ligated NEP-ARRs (or PSA-ARRs) were inserted into HindIII site of ptkCAT linearized with HindIII restriction enzyme. PNAR-RAREtkCAT was constructed by first linearizing plasmid p1AREtkCAT with HindIII restriction enzyme and inserting multimerized NEP-ARRs oligonucleotides producing pNARRAREtkCAT (two copies of NEP-ARR in front of one copy NEP-ARE).

2.5. Transfection and measurement of CAT activities

Plasmid DNA (6 μg) were transfected (with or without co-transfection of 0.16 μg of AR expression vector DNA) (Palvimo et al., 1993) using Lipofectamin™ (GIBCO-BRL) according to the manufacturer's recommendations. After 24 h, cells were trypsinized and equal numbers of cells were plated in four wells of a six-well tissue culture plate (FALCON 3502 tissue culture plate). Two wells were incubated in media containing 30 nM steroid for 24 h and two in media without steroid (control). Cells were harvested, and cell lysates prepared for performing CAT assays using a CAT enzyme assay system (PROMEGA, Madison, WI). The plasmid ptkCAT (containing tk promoter only) was used as a negative control and pGREtkCAT (containing a consensus GRE upstream of the tk promoter (Schena et al., 1989)) was used as a positive control. Each transfection experiment was performed in duplicate on at least three separate occasions.

2.6. DNase-I footprinting analysis

DNase-I footprinting was performed using the Core Footprinting Kit (Stratagene) essentially as described (Freedman et al., 1988). The plasmid containing the NEP type II promoter (532 bp, −386/+146) in PXP2 vector (Ishimaru and Shipp, 1995) was restriction digested with HindIII and labeled with ³²P using a T4 kinase and then digested with XbaI. The ³²P labeled 532

bp DNA fragment was purified on a polyacrylamide gel, incubated with 100 μg nuclear extracts per reaction, digested with DNase-I, and then run on a 6% polyacrylamide/urea denaturing sequencing gel along side a G specific Maxim Gilbert reaction of the identical labeled DNA fragment.

3. Results

3.1. The presence of an ARE consensus sequence in exon 24 of the NEP gene

A search for a putative androgen response element using the TFSEARCH program identified within exon 24 of the NEP gene a sequence of 85.5% identity with a consensus HRE. We designated this putative cis-element which lies in the 3′-untranslated region as NEP-ARE. For comparison of this NEP-ARE, the consensus ARE and AREs in several other genes are shown in Table 1. The AR recognition sequence that constitutes a specific ARE contains two 6-bp asymmetrical half-sites separated by a 3-bp spacer (Roche et al., 1992). The right half-site of the putative NEP-ARE was a perfect match with the right half-site of the Slp (HRE 2), and in the left half-site, all the bases except for 3C appeared in other the AREs. Therefore, this sequence was a strong candidate as a DNA target for AR.

3.2. Interaction of the NEP-ARE with AR in nuclear extracts and AR proteins

Binding of AR protein to the NEP-ARE in vitro was examined by mobility shift assays. Fig. 1A shows that relative to the labeled NEP-ARE oligonucleotide in the absence of protein (lane 1) or in the presence of COS-7 cell nuclear extracts (lane 2), retarded mobility only occurred in the presence of nuclear extracts from COS-7 cells transiently transfected with AR (COS-7/AR) (lane 3). This shift, indicative of binding AR protein to

the oligonucleotide which was identical to binding observed using consensus ARE oligonucleotides (not shown), was eliminated by binding with 100-fold excess unlabeled NEP-ARE or rat consensus ARE (rARE) oligonucleotides (lanes 4 and 5), which shows that the complexes formed between DNA and nuclear proteins are sequence-specific. To identify the nature of the above-given DNA-protein complexes, radio-labeled NEP-AREs were incubated with rabbit pre-immunoserum or an anti-AR antibody together with nuclear extracts from COS-7/AR cells. While non-immune serum had little effect on the band-shift profile (lane 6), the anti-AR antibodies shifted the AR/DNA complex to a position in the gel indicative of a larger sized complex (lane 7). Experiments with nuclear extract prepared from LNCaP prostate cancer cells which express AR showed that retarded mobility occurred in the presence of LNCaP nuclear extracts (Fig. 1B, lane 1) which increased in intensity when cells were incubated in DHT (lane 4). The complexes formed between AR and NEP-ARE in the absence of DHT could be displaced by 100-fold excess unlabeled NEP-ARE (lane 2) or rARE (lane 3) oligonucleotides. Taken together, these data suggest that the gel retardation of the NEP-ARE is a consequence of an interaction with AR.

To further demonstrate the interaction between NEP-ARE and AR protein, a gel mobility shift assay was performed with purified AR protein (Fig. 1C). Purified AR proteins could interact with labeled NEP-ARE (lane 2). The DNA-protein complexes did not occur in the absence of protein (lane 1), and could be displaced by 100-fold excess unlabeled NEP-ARE oligonucleotides (lane 3). A mutated NEP-ARE was constructed (Table 1) based on crystal structure data of the GR DNA-binding domain complexed with DNA which indicated which nucleotides were critical for specific GR (or similar steroid hormones) binding to a GRE-like consensus element (Luisi et al., 1991). Incubation with 100-fold excess mutated NEP-ARE oligonucleotides could only partially inhibit AR bind-

Table 1
Sequence comparison of the NEP-ARE with ARE elements in other genes

| Gene	Species	Sequence	Location	Reference
NEP-ARE	Human	GTCACAaagAGTTCT	EXON 24	
Mutated NEP-ARE		GTCGTAaagACATTT		
Consensus ARE		GGTACAnnnTGTTCT		(Roche et al., 1992)
PSA (uARE)	Human	GGAACAtatTGTATC	−4145/−4136	(Schuur et al., 1996)
PSA (pARE)	Human	AGAACAgcaAGTGCT	−177/−163	(Riegman et al., 1991; Murtha et al., 1993)
C3(1)	Rat	AGTACGtcaTGTTCT	1st intron	(Tan et al., 1992
Slp (HRE-1)	Mouse	GTAATTatcTGTTCT	−126/−112	(Adler et al., 1991)
Slp (HRE-2)	Mouse	TGGTCAgccAGTTCT	−143/−128	(Adler et al., 1991)
Slp (HRE-3)	Mouse	AGAACAggcTGTTTC	−158/−144	(Adler et al., 1991)
KLK-2	Human	GGAACAgcaAGTGCT	−170/−156	(Riegman et al., 1991; Murtha et al., 1993)
MVDP	Mouse	TGAAGTtccTGTTCT	−111/−97	(Fabre et al., 1994)
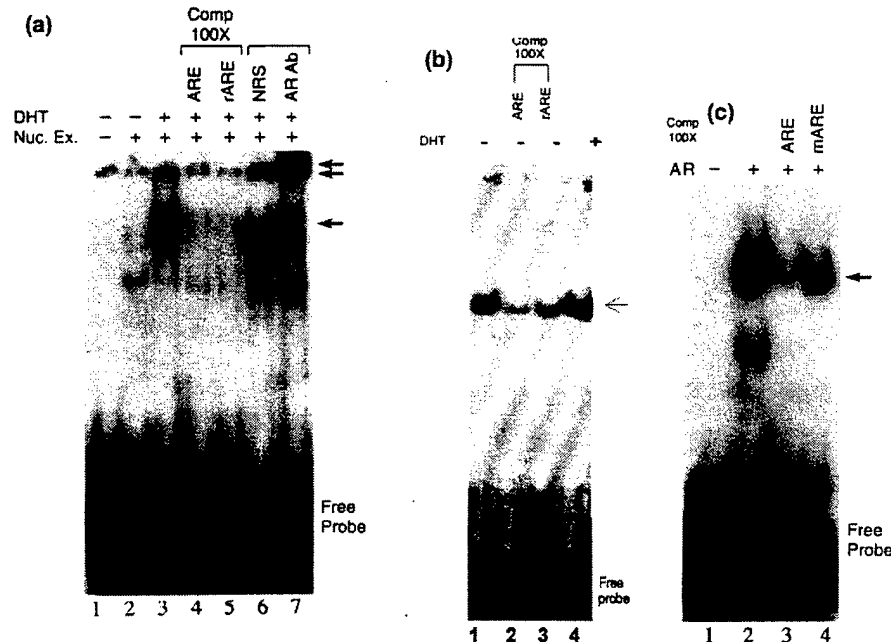
Fig. 1. Gel mobility shift assay of NEP-ARE with nuclear extracts and competition analysis. (A) Radiolabled NEP-ARE was incubated with nuclear extracts from COS-7 and COS-7/AR cells (30 nM DHT) and separated on a 4% polyacrylamide gel. Lane 1, no extract; lane 2, COS-7; Lane 3, COS-7/AR cells; Lane 4, COS-7/AR cells + 100-fold excess unlabled NEP-ARE; Lane 5, COS-7/AR cells + 100-fold excess unlabled consensus rat ARE (rARE). Lane 6, COS-7/AR cells + DHT + normal rabbit serum (NRS); Lane 7, COS-7/AR cells + DHT + antibody which recognizes AR (AR Ab) (Santa Cruz Biotechnology Inc.). Note mobility is partially impeded in lane 7 (upper band). Arrow indicates the complex formed between NEP-ARE and AR protein in nuclear extracts from COS-7/AR cells; double arrow indicates supershift band formed between the AR Ab and AR protein. Nuc.Ex., nuclear extract; DHT, dihydrotestosterone; Comp, competitor. (B) Radiolabled NEP-ARE was incubated with the nuclear extracts from LNCaP cells treated without (lanes 1, 2 and 3) or with 30 nM DHT (lane 4) and competed with 100-fold excess unlabled NEP-ARE or rat ARE (rARE) (lanes 2 and 3), then resolved on a 4% polyacrylamide gel. Arrow indicates the complex formed between NEP-ARE and AR in nuclear extracts from LNCaP cells. (C) Radiolabled NEP-ARE was incubated with purified AR and separated on a 4% polyacrylamide gel. Lane 1, no protein; lane 2, purified AR protein; Lane 3, purified AR protein + 100-fold excess unlabled NEP-ARE; Lane 4, purified AR + 100-fold excess unlabled mutated NEP-ARE (mARE). Arrow indicates the complexes formed between the NEP-ARE and AR proteins.

ing to wild-type NEP-ARE oligonucleotides (Fig. 1C, lane 4). These data confirm that the NEP-ARE specifically binds to AR.

3.3. NEP-ARE can confer androgen inducibility

To test the ability of the NEP-ARE as a target for AR mediated transcription, the plasmid p3NAREtkCAT containing three copies of NEP-ARE was co-transfected with an AR expression plasmid into CV-1 cells, and CAT activities measured. pGREtkCAT (containing a consensus GRE sequence which confers glucocorticoid and androgen inducibility) and ptkCAT (containing the tk promoter only) were transfected into CV-1 cells as positive control and negative control, respectively. As illustrated in Fig. 2, addition of DHT to cells with p3NAREtkCAT resulted in a ∼4-fold increase in CAT activity, compared with 7–8-fold with pGRE-tkCAT, and no induction with ptkCAT. These results show that in living cells AR binds to the NEP-ARE, and that this interaction results in androgen dependent transcription.

3.4. The interaction of GR and PR proteins with NEP-ARE and induction by dexamethasone and progesterone

To evaluate whether the NEP-ARE also interacted with other related steroid hormone receptors, we performed in vitro binding studies with partially purified GR(DBD) and PR proteins. As shown in Fig. 3, a mobility retarded band appeared with purified GR (Fig. 3A, lane 2) or PR proteins (Fig. 3B, lane 2). These DNA-protein complexes could be displaced by 100-fold excess unlabeled NEP-ARE oligonucleotide (Fig. 3A and B, lane 3), but not by excess unlabeled mutated NEP-ARE (Fig. 3A and B, lane 4). To confirm the interaction of the NEP-ARE with GR and PR, the p3NAREtkCAT plasmid was co-transfected with GR or PR expression vectors into CV-1 cells. Treatment with 30 nM dexamethasone or progesterone resulted in a seven- and three-fold induction of CAT activities, respectively (Fig. 4). These data indicate that NEP-ARE can also mediate induction by glucocorticoid and progesterone.

3.5. Functional differences between the NEP-ARE, mutated NEP-ARE(M) and NEP-ARE(L) with extended 5' and 3' flanking regions

The sequence adjacent to a receptor binding site may create composite activation elements that effect steroid binding. To study the potential influence of the base composition of the flanking regions surrounding the NEP-ARE core, we compared the steroid induction mediated by NEP-ARE(M) (containing a site-directed mutation in the core region) in pNARE(M)tkCAT and NEP-ARE(L) (with extended 5'- and 3'-flanking region) in Pnare(L)tkCAT to the NEP-ARE in p1NAREtkCAT (containing one copy of NEPARE) and p3NAREtkCAT (containing three copies of NE-PARE). The extended flanking region did not contain any additional potential ARE sequences. These constructs were studied in CV-1 cells following co-transfection of these plasmids with AR, GR or PR expression vectors (Fig. 5). Steroid induction of CAT activities following incubation with either DHT, dexamethasone or progesterone was higher for reporter plasmids with three copies of the NEP-ARE than with a single copy. Mutation of the NEP-ARE resulted in a loss of steroid inducibility. The fold inductions of CAT activities by all three steroid hormones using the NEP-ARE(L) which contains one copy of extended 5'-and 3'-flanking region of the core region of NEP-ARE were similar to those observed using the identical plasmid containing

one copy of the NEP-ARE (p1NAREtkCAT). These results demonstrate that the sequences in the core region, but not the 203 bp of 5'- or 75 bp of 3'- flanking region of NEP-ARE are required to mediate steroid hormone induction.

3.6. Identification of a second androgen responsive region by DNase-I footprinting in the NEP promoter

The NEP-ARE is located in the 3'-UTR of the NEP gene. To identify other potential AR related nuclear factor binding sites located in the NEP promoter, DNase I footprinting assays were performed in the promoter region. We analyzed the region −386 to +146 covering the type II promoter of NEP gene as this region has previously been shown to possess the highest degree of endogenous promoter activity and the cis-elements responsible for tissue specific expression of the NEP gene Li et al., 1995). DNase I cleavage patterns protected by crude nuclear extracts from COS-7 and COS-7(AR) cells were compared (Fig. 6A). A weakly protected region (−305/−283) was detected using COS-7/AR extracts (lane 3) not observed with COS-7 extracts (lane 2). The sequence of this protected region (GGTGCGGGTCGGAGGGATGCCCA) did not contain a typical ARE motif, and was designated NEP-ARR (androgen responsive region). Comparison of this sequence with other reported androgen binding site sequences revealed 65.2% homology to an ARR
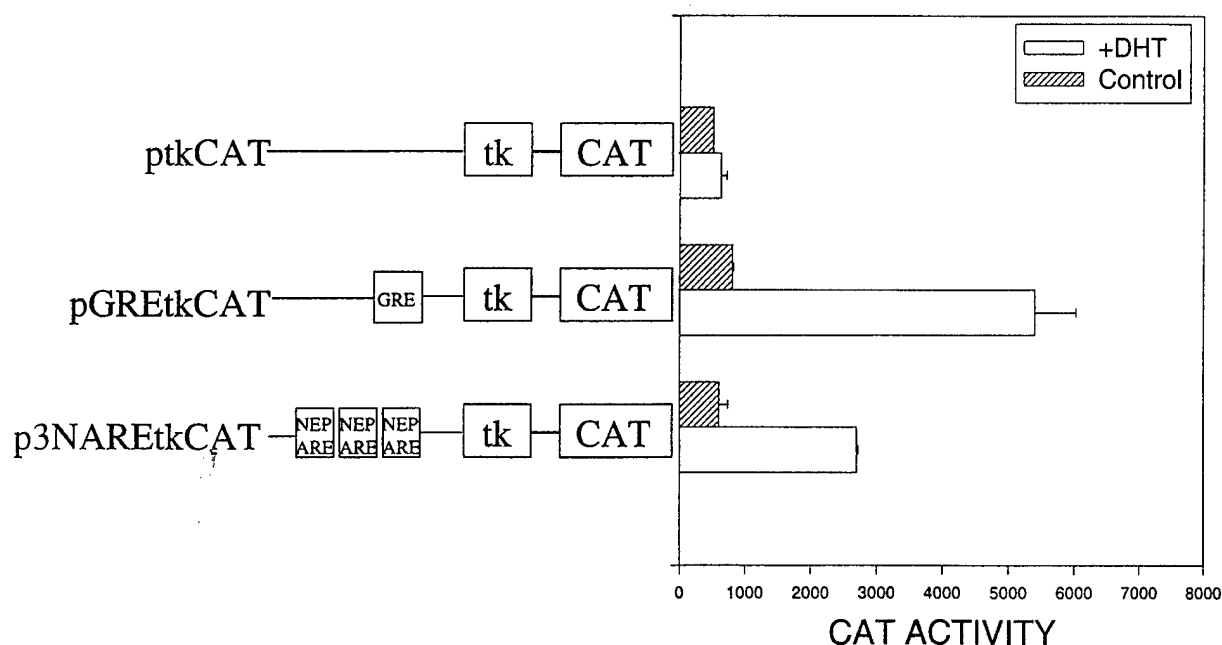

Fig. 2. CAT assays showing the androgen induction mediated by the NEP-ARE element CV-1 cells were co-transfected with either ptkCAT, pGRE-tkCAT or p3NAREtkCAT together with an AR expression plasmid. Following overnight incubation, transfected cells were incubated in the presence or absence of 30 nM DHT for 24 h. Results are representative of one set of the experiments performed in duplicate. Each experiment was performed on at least three separate occasions. Student's t-test; ptkCAT, $P = 0.03$; pGRE-tkCAT, $P = 0.02$; p3NAREtkCAT, $P = 0.004$.
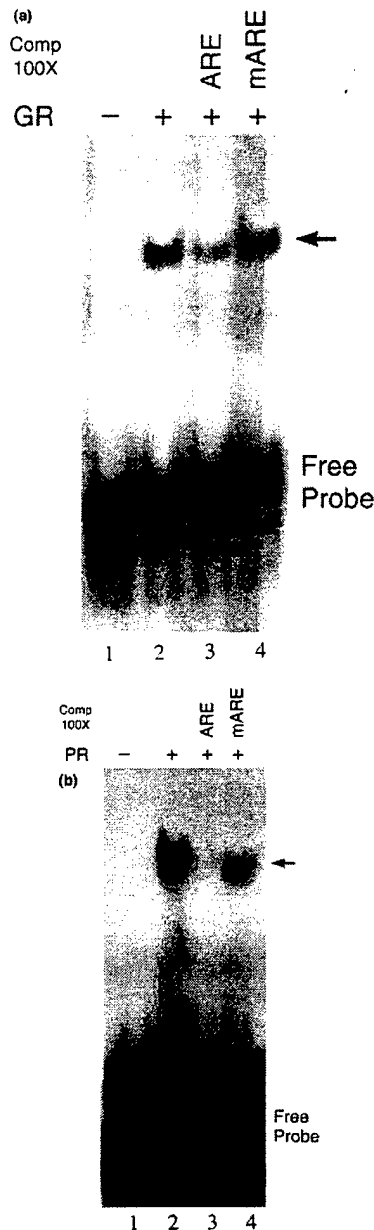
Fig. 3. The interaction of the NEP-ARE with purified GR and PR proteins The NEP-ARE oligonucleotide was used to perform gel shift assays with purified proteins of glucocorticoid receptor (A) and progesterone receptor (B), respectively. (−), no protein; (+), with protein. Unlabled oligonucleotides used for competition assays are 100-fold excess unlabeled NEP-ARE (lane 3) or mARE (mutated NEP-ARE; Lane 4). Arrows indicate the complexes formed between the NEP-ARE and purified GR(DBD) and PR proteins.

3.7. Interaction of the NEP-ARR with AR but not GR proteins

Binding of AR protein to the NEP-ARR and the PSA-ARR in vitro was examined by mobility shift assays. Purified AR proteins could interact with labeled NEP-ARR (Fig. 7A, lane 2) or PSA-ARR (Fig. 7B, lane 2). The DNA-protein complexes did not occur in the absence of protein (Fig. 7A and B, lane 1), and could be displaced by 100-fold excess unlabeled NEP-ARR (Fig. 7A and B, lane 3), PSA-ARR (Fig. 7A and B, lane 4) and NEP-ARE oligonucleotides (Fig. 7A and B, lane 5). As shown in Fig. 7C, in contrast to the NEP-ARE which bound GR(DBD) (lane 2), GR did not bind to the NEP-ARR (lane 4) and weakly bound to the PSA-ARR (lane 6). The complexes formed between GR protein and the NEP-ARE could be competed with 100-fold excess unlabeled NEP-ARE (Fig. 7D, lane 5), but not with 100-fold excess unlabeled NEP-ARR (lane 3) or PSA-ARR (lane 4). Similar results were obtained using full-length GR protein (Fig. 7E). Complexes formed between NEP-ARE and GR protein (lane 2) which could be competed with 100-fold excess unlabeled NEP-ARE (lane 3). No complexes formed between full-length GR protein and NEP-ARR (lane 5). Taken together, these data suggest that the NEP-ARR located in the NEP promoter is a cis-element that specifically interacts with AR but not with the GR proteins.
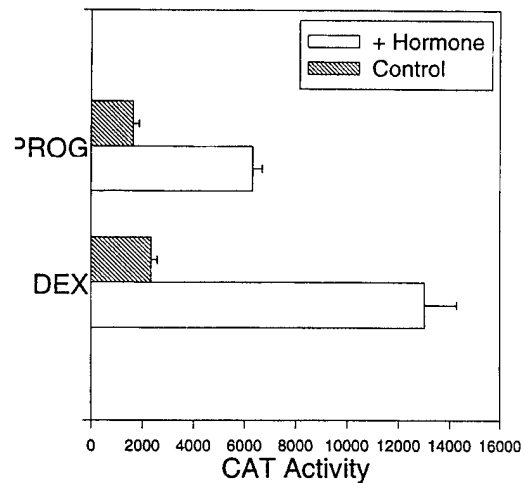

Fig. 4. Steroid hormone induction of pNAREtkCAT activities. CV-1 cells were co-transfected with p3NAREtkCAT together with a GR or PR expression plasmid. Following overnight incubation, transfected cells were incubated in the presence or absence of 30 nM PROG (progesterone) or DEX (dexamethasone) for 24 h. Results are representative of one set of the experiments performed in duplicate. Each experiment was performed on at least three separate occasions. Student's t-test, PROG, $P = 0.009$; DEX, $P = 0.01$.

previously identified in the promoter of the prostate specific antigen (PSA) gene, designated as PSA-ARR (Cleutjens et al., 1996) (Fig. 6B).
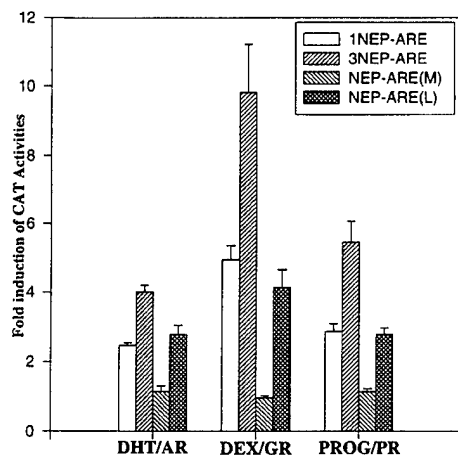
Fig. 5. The influence of flanking and core regions of the NEP-ARE on its biological function. The fold inductions of CAT activities (CAT activities in the presence of steroid hormones/CAT activities in the absence of steroid hormones) were obtained by co-transfection of plasmids p1NAREtkCAT (one copy of NEP-ARE), p3NAREtkCAT (three copies of NEP-ARE), pNARE(M)tkCAT (one copy of mutated NEP-ARE), and pNARE(L)tkCAT (one copy of NEP-ARE surrounded by 5′ and 3′ flanking regions) with AR, GR or PR expression vectors in CV-1 cells. Following overnight incubation, transfected cells were incubated in the presence or absence of 30 nM DHT. DEX (dexamethasone) and PROG (progesterone) for 24 h. Results representative of one experiment performed in triplicate. Fold induction — Students t-test P value: DHT: 1NEP-ARE: 2.5 ($P =$ 0.003); 3NEP-ARE: 4.0 ($P = 0.004$); NEP-ARE(M): 1.2 ($P = 0.35$); NEP-ARE(L): 2.8 ($P = 0.01$); DEX: 1NEP-ARE: 4.9 ($P = 0.001$); 3NEP-ARE: 9.8 ($P = 0.001$); NEP-ARE(M): 0.97 ($P = 0.62$); NEP-ARE(L): 4.1 ($P = 0.002$); PROG: 1NEP-ARE: 2.9 ($P = 0.001$); 3NEP-ARE: 5.5 ($P = 0.008$); NEP-ARE(M): 1.2 ($P = 0.17$); NEP-ARE(L): 2.8 ($P = 0.001$).

3.8. Additive effects of NEP-ARR and NEP-ARE in androgen inducibility in PC cells

To test the ability of the NEP-ARR as a target for AR mediated transcription, the plasmids pNEPAR-RAREtkCAT containing two copies of the NEP-ARR and one copy of the NEP-ARE, and pNEP-AREtkCAT containing one copy of the NEP-ARE were each co-transfected with an AR expression plasmid into PC-3/AR cells and CAT activities measured following incubation in 30 nM DHT. The addition of two copies of ARR in front of one copy of NEP-ARE results in an increase of the induction folds of CAT

activity [from 2.0 (in pNEP-AREtkCAT) to 4.7 (in pNEPARRAREtkCAT)] (Fig. 8A). To test steroid induction mediated by the NEP-ARR, p3NEPARRtkCAT was co-transfected with AR, GR and PR expression vectors into PC-3/AR cells. In contrast to the 2–3-fold induction by DHT, no induction mediated by NEP-ARR with dexamethasone and progesterone could be detected (Fig. 8B). These data demonstrate that NEP-ARR is a functional cis-element which can mediate induction by androgen but not dexamethasone and progesterone in the PC cells.

4. Discussion

We report the identification and initial characterization of an ARE located in the 3′ end of the NEP gene. The sequence of this element is similar to the previously reported HREs which regulate steroid hormone responsiveness of a variety of genes, including PSA, however those elements are predominantly located in the 5′-flanking region of the transcription initiation site. The NEP-ARE is found in exon 24 of the NEP gene. This exon is ~3400 bp in length and encodes the COOH terminus of the protein (amino acids 717–749), the translation stop codon and the 3′ untranslated region (UTR) (D'Adamio et al., 1989). Although AREs have been identified in intron sequences (Claessens et al., 1989; Ho et al., 1993; Avellar et al., 1997), to the best of our knowledge this is the first report of the identification of a functional ARE in the 3′-UTR.

The evidence indicating the presence of the NEP-ARE is as follows (1) DHT induces transcription of NEP in LNCaP cells (Papandreou et al., 1998); (2) the NEP-ARE sequence is homologous to the sequence of previously reported AREs (Table 1) (Roche et al., 1992); (3) the NEP-ARE specifically complexes with nuclear extracts from AR-expressing cells and with purified AR; and (4) the NEP-ARE mediates androgen-dependent enhancement of transcription in CV-1/AR and PC-3/AR cells. The NEP-ARE also interacts with GR and PR, and can mediate dexamethasone and progesterone dependent enhancement of transcription, similar to the other AREs which act as steroid hormone response elements in similar assays of steroid hormone responsiveness. These results are consistent with the
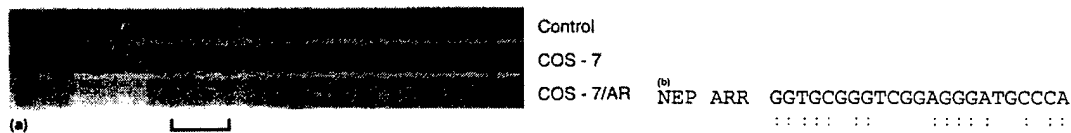

Control
COS - 7
COS - 7/AR   NEP ARR   GGTGCGGGTCGGAGGGATGCCCA
                        : : : : :   : :   : : : : :   : : :
             PSA ARR   GGTGCAGGGATCAGGGAGTCTCA

Fig. 6. DNA Footprinting analysis of the NEP promoter. (A) A 532 bp promoter DNA fragment was labeled at the 5′ end, incubated with no nuclear proteins (Lane 1), COS-7 nuclear extract (Lane 2) or COS-7/AR nuclear extract (Lane 3) and digested with DNase-I. The region −283/−305 (bracketed) relative to transcription initiation site was protected by nuclear extracts from COS-7/AR but not from COS-7 cells. (B) The 23 bp protected sequence is 65.2% homologous to an ARR element previously identified in the PSA promoter.
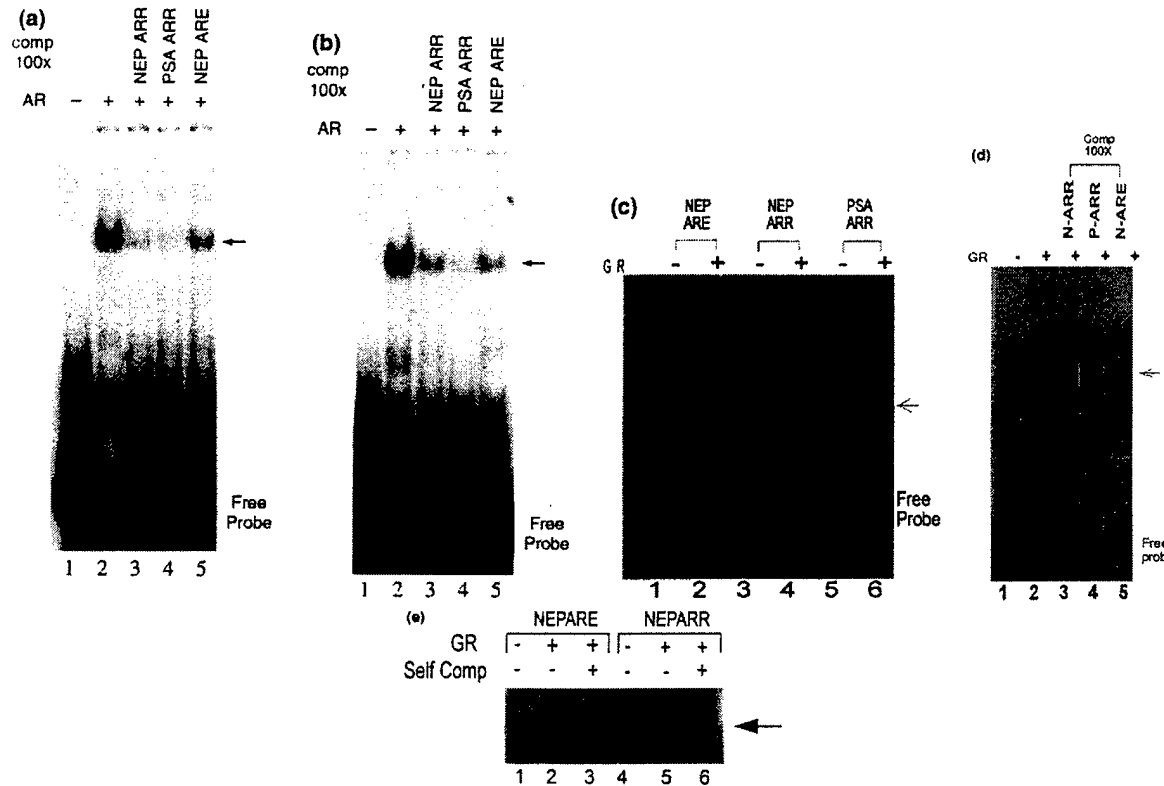
Fig. 7. Interaction of NEP-ARR and PSA-ARR with purified AR and GR proteins. Radiolabeled NEP-ARR (A) or PSA-ARR (B) was incubated with purified AR proteins and separated on a 4% polyacrylamide gel. DNA-protein complexes were compared with 100-fold excess unlabeled NEP-ARR, PSA-ARR or NEP-ARE oligonucleotides. Arrow indicates the complexes formed between purified AR and oligonucleotides (NEP-ARR or PSA-ARR). (C) Radiolabeled NEP-ARE (lanes 1 and 2), NEP-ARR (lanes 3 and 4) and PSA-ARR (lanes 5 and 6) were incubated without (lanes 1, 3 and 5) or with purified GR(DBD) (DNA binding domain) (lanes 2, 4 and 6) and then separated on a 4% polyacrylcmide gel. Arrow indicates the complexes formed between GR(DBD) and oligonucleotides. (D) Radiolabled NEP-ARE was incubated without (lane 1) or with purified GR (DBD) proteins (lanes 2, 3, 4, and 5) and then separated on a 4% polyacrylcmide gel. In lanes 3, 4 and 5, 100-fold excess unlabled NEP-ARR, PSA-ARR or NEP-ARE oligonucleotides were added as competitors, respectively. Arrow indicates the complexes formed between oligonucleotides and GR (DBD) proteins. (E) Radiolabled NEP-ARE (lanes 1–3) and NEP-ARR (lanes 4–6) were incubated without (lanes 1 and 4) or with purified GR proteins (lanes 2, 3, 5, and 6 and then separated on a 4% polyacrylcmide gel. One hundred-fold excess unlabled NEP-ARE (lane 3) or NEP-ARR (lane 6) oligonucleotides were added as competitors. Arrow indicates the complexes formed between GR proteins and NEP-ARE oligonucleotides but not with NEP-ARR oligonucleotides. Only upper part of the gel is shown.

previous reports which showed that NEP gene expression can be enhanced by progesterone (Casey et al., 1991) and glucocorticoids (Borson and Gruenert, 1991; Graf et al., 1998; van der Velden et al., 1998), suggesting that steroid hormone regulation of the NEP gene occurs in a cell context specific manner.

The fold-enhancement of NEP-ARE mediated transcription by DHT is low as compared with other previously reported elements, implying that other cis-elements are required for its full function. More than one ARE is frequently required to fully mediate androgen responsiveness as observed in the probasin and PSA genes (Rennie et al., 1993; Cleutjens et al., 1996). Recent studies also indicate that simple (consensus) AREs are often part of a more complicated hormone-responsive unit or complex ARE (Lindzey et al., 1994). For instance, the Slp gene contains a complex ARE composed of a core ARE and adjacent enhancers

which are two degenerated hormone response elements that do not function independently but synergize with the core ARE sequence (Lindzey et al., 1994). Similarly, the glucocorticoid response unit in carbamoylphosphate synthetase I gene contains several other cis- and trans-elements (C/EBP, HNF3 and protein kinase A) in addition to the GRE element which are required for the full function of the steroid receptor (Christoffels et al., 1998). Examination of the flanking sequences around the core region of the NEP-ARE sequence did not detect any enhancers in 292 bp extended 5'- and 3'-flanking region.

In addition to the NEP-ARE, we also identified a second sequence in the NEP type 2 promoter by DNA footprint analysis which selectively bound AR but not GR proteins, and induced transcription in a reporter vector transfected into PC cells in response to incubation with DHT but not other steroid hormones. Al-

though the AR proteins strongly interacted with the NEP-ARR, the fold enhancement of transcription in a thymidine kinase promoter was only ~ two-fold. This weak induction is similar to that observed with a homologous element identified in the promoter of the PSA gene. Like the PSA-ARR, one copy of the NEP-ARR upstream of the TK promoter weakly induced pro-
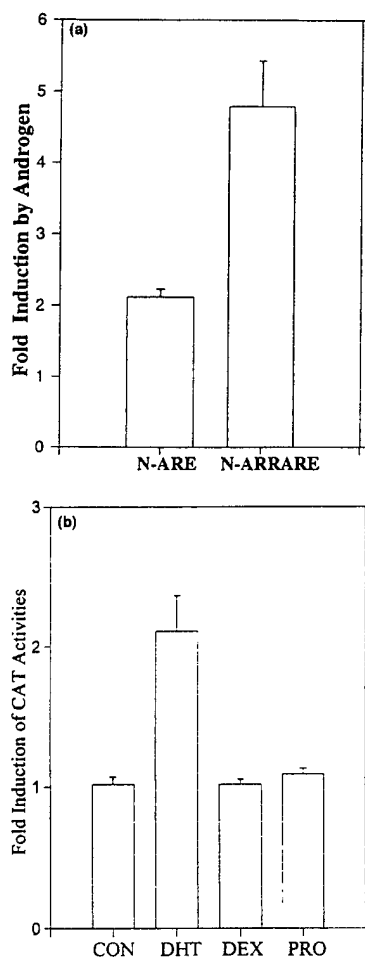

Fig. 8. Functional analysis of NEP-ARR. (A) Additive effect of NEP-ARR in androgen induction. The plasmids pN-AREtkCAT and pN-ARRAREtkCAT were transfected into PC-3/AR cells, respectively. CAT activities were determined after 24 h incubation in media containing 30 nM DHT. Student's t-test: N-ARE vs. control: $P = 0.08$; N-ARRARE vs. control: $P = 0.006$; and N-ARRARE vs. N-ARE: $P = 0.05$. (B) Steroid induction mediated by NEP-ARR. Plasmid p3ARRtkCAT was co-transfected into PC-3/AR cells together with AR, GR and PR expression vectors. CAT activities were determined after 24 h incubation in media containing (+ steroid) or without (− steroid) 30 nM DHT (DHT), dexamethasone (DEX) and progesterone (PRO), respectively. Fold-induction of CAT activities shown. CON: without DNA transfection. Student's t-test: $P = 0.870$(CON), $P = 0.069$ (DHT), $P = 0.701$(DEX) and $P = 0.218$(PRO). Greater than 10-fold induction of CAT activity was observed in PC-3/AR cells co-transfected with pGREtkCAT positive control vector and AR, GR or PR expression vectors following steroid hormone treatment (not shown).

moter activity by androgen (Cleutjens et al., 1996). Cleutjens et al. proposed that the PSA-ARR was a low affinity AR containing a degenerated palindromic sequence with homology to the consensus ARE in 6 out of 12 positions (Cleutjens et al., 1996) (Fig. 9). However, in the PSA-ARR, point mutations at position 5 of the left half-site (C–G) or of position 2 of the right half-site (G–C) blocked binding of the PSA-ARR oligonucleotide to AR(DBD). Both of these nucleotides are not conserved in the NEP-ARR (Fig. 9), suggesting that either these two elements are not related, or that that AR binding to this sequence involves other homologous nucleotides. One possibility is the G/CAGGGA sequence present in the NEP-ARR once and twice in the PSA-ARR. Direct repeats of GRE-like core motifs can function as a hormone response element (Zhou et al., 1997). In addition, the sequence CA GGGACAnnn and CT GGGACAnnn as left half-sites have previously been shown to bind AR (Roche et al., 1992). Preliminary studies indicate that partial deletion or mutation of the CAGGGA element inhibits binding of the NEP-ARR to AR (unpublished data), suggesting that this sequence may possess some intrinsic AR binding capability.

In a thymidine kinase promoter, the one copy of the NEP-ARE and two copies of the NEP-ARR interact to stimulate a five-fold increase in promoter activity, compared with an approximate two-fold increase with either one copy of the NEP-ARE or two copies of the NEP-ARR. This cooperation is additive and not synergistic as observed in similar studies of more than one androgen responsive region isolated from other hormone responsive genes. Another ARE or enhancer of androgen responsiveness may be located far upstream of the NEP promoter as observed in the PSA gene (Cleutjens et al., 1997; Pang et al., 1999). Recent studies also highlight the involvement of other factors in the activation of AR by androgen following binding of the activated receptor to an ARE. A variety of nuclear proteins are now recognized to interact with steroid hormone receptors. These include GTPs (general transcription proteins), sequence-specific transcription factors, co-activators, and chromatin-related factors (Beato and Sanchez-Pacheco, 1996). The involvement of numerous factors underline the complexity of the molecular mechanism involved in transactivation by hormone receptors (Beato and Sanchez-Pacheco, 1996).

In conclusion, the regulation of NEP gene transcription has not been well characterized and appears complex. Despite the presence of multi-promoters with multiple transcription initiation sites and unique 5' untranslated sequences, there is a common coding sequence and all transcripts encode the identical protein. We have begun to decipher steroid regulation of NEP expression by identifying an ARE in the 3'-UTR and a novel androgen responsive region in the NEP promoter.

Consensus ARE	GGTACA nnn TGTTCT	
PSA ARR	GGTGCAGGGATGA GGG AGTCTCA	GGTGCAGGGATCAGGGAGTCTCA
NEP ARR	GGTGCGGGTCGGA GGG ATGCCCA	GGTGCGGGTCGGAGGGATGCCCA

Fig. 9. Comparison of NEP-ARR with PSA-ARR. Degenerate ARE proposed in PSA-ARR is compared with homologous region in the NEP-ARR. Note that mutations in the PSA-ARR of position 5 (shaded) in the left half site from C to G and of position 2 (shaded) in the right half site from G to C were reported to result in loss of binding to AR(DBD) (Cleutjens et al., 1996). Right column shows areas of conserved homology between PSA-ARR and NEP-ARR.

These elements are probably part of a larger complex which regulates the NEP gene expression in prostate and other hormonally responsive cells.

References

Adler, A.J., Scheller, A., Hoffman, Y., Robins, D.M., 1991. Multiple components of a complex androgen-dependent enhancer. Mol. Endocrinol. 5, 1587–1596.

Aprikian, A.G., Han, K., Guy, L., Landry, F., Begin, L.R., Chevalier, S., 1998. Neuroendocrine differentiation and the bombesib/gastrin releasing peptide family of neuropeptides in the progresssion of human prostate cancer. Prostate 8, 52–61.

Avellar, M.C., Gregory, C.W., Power, S.G., French, F.S., 1997. Androgen-dependent protein interactions within an intron 1 regulatory region of the 20-kDa protein gene. J. Biol. Chem. 272, 17623–17631.

Beato, M., Sanchez-Pacheco, A., 1996. Interaction of steroid hormone receptors with the transcription initiation complex. Endocrine Rev. 17, 587–609.

Borson, D.B., Gruenert, D.C., 1991. Glucocorticoids induce neutral endopeptidase in transformed human tracheal epithelial cells. Am. J. Physiol. 260, L83–L89.

Casey, M.L., Smith, J.W., Nagai, K., Hersh, L.B., MacDonald, P.C., 1991. Progesterone-regulated cyclic modulation of membrane metalloendopeptidase (enkephalinase) in human endometrium. J. Biol. Chem. 34, 23041–23047.

Christoffels, V.M., Grange, T., Kaestner, K.H., Cole, T.J., Darlington, G.J., Croniger, C.M., Lamers, W.H., 1998. Glucocorticoid receptor, C/EBP, HNF3, and protein kinase A coordinately activate the glucocorticoid response unit of the carbamoylphosphate synthetase I gene. Mol. Cell. Biol. 18, 6305–6315.

Claessens, F., Celis, L., Peeters, B., Heyns, W., Verhoeven, G., Rombauts, W., 1989. Functional characterization of an androgen response element in the first intron of the C3(1) gene of prostatic binding protein. Biochem. Biophys. Res. Commun. 164, 833–840.

Cleutjens, K., van Eekelen, C., van der Korput, J.G.M., Brinkmann, A.O., Trapman, J., 1996. Two androgen response regions cooperate in steroid hormone regulated activity of the prostate specific antigen promoter. J. Biol. Chem. 271, 6379–6388.

Cleutjens, K.B., van der Korput, H.A., van Eekelen, C.C., van Rooij, H.C., Faber, P.W., Trapman, J., 1997. An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter. Mol. Endocrinol. 11, 148–161.

D'Adamio, L., Shipp, M.A., Masteller, E.L., Reinherz, E.L., 1989. Organization of the gene encoding common acute lymphoblastic leukemia antigen (neutral endopeptidase 24.11): multiple miniexons and separate 5' untranslated regions. Proc. Natl. Acad. Sci. USA 86, 7103–7107.

Dai, J.L., Maiorino, C.A., Gkonos, P.J., Burnstein, K.L., 1996. Androgenic up-regulation of androgen receptor cDNA expression in androgen-independent prostate cancer cells. Steroids 61, 531–539.

Fabre, S., Manin, M., Pailhoux, E., Veyssiere, G., Jean, C., 1994. Identification of a functional androgen response element in the promoter of the gene for the androgen-regulated aldose reductase-like protein specific to the mouse vas deferens. J. Biol. Chem. 269, 5857–5864.

Freedman, L.P., Alroy, I., 1993. Uses of the gel mobility shift assay to study nuclear hormone receptor-DNA interactions. In: Adolph, K. (Ed.), Methods in Molecular Genetics. Academic Press, New York, pp. 280–299.

Freedman, L.P., Luisi, B.F., Korszun, Z.R., Basavapa, R., Sigler, P.B., Yamamoto, K.R., 1988. The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. Nature 334, 543–546.

Graf, K., Schaper, C., Grafe, M., Fleck, E., Kunkel, G., 1998. Glucocorticoids and protein kinase C regulate neutral endopeptidase 24.11 in human vascular smooth muscle cells. Basic Res. Cardiol. 93, 11–17.

Ho, K.C., Marschke, K.B., Tan, J., Power, S.G., Wilson, E.M., French, F.S., 1993. A complex response element in intron 1 of the androgen-regulated 20-kDa protein gene displays cell type-dependent androgen receptor specificity. J. Biol. Chem. 268, 27226–27235.

Ishimaru, F., Mari, B., Shipp, M.A., 1997. The type 2 CD10/neutral endopeptidase 24.11 promoter: functional characterization and tissue-specific regulation by CBF/NF-Y isoforms. Blood 89, 4136–4145.

Ishimaru, F., Shipp, M.A., 1995. Analysis of the human CD10/neutral endopeptidase 24.11 promotor region: two separate regulatory elements. Blood 85, 3199–3207.

Li, C.W., Booze, R.M., Hersh, L.B., 1995. Tissue-specific expression of rat neutral endopeptidase (neprilysin) mRNAs. J. Biol. Chem. 270, 5723–5728.

Lindzey, J., Kumar, M.V., Grossman, M., Young, C., Tindall, D.J., 1994. Molecular mechanisms of androgen action. Vitamins and Hormones 49, 383–432.

Luisi, B.F., Xu, W.X., Otwinowski, Z., Freedman, L.P., Yamamoto, K.R., Sigler, P.B., 1991. Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature 352, 497–505.

Mader, S., Leroy, P., Chen, J.Y., Chambon, P., 1993. Multiple parameters control the selectivity of nuclear receptors for their response elements. Selectivity and promiscuity in response element recognition by retinoic acid receptors and retinoid X receptors. J. Biol. Chem. 268, 591–600.

Murtha, P., Tindall, D.J., Young, C.Y., 1993. Androgen induction of a human prostate-specific kallikrein, hKLK2: characterization of an androgen response element in the 5' promoter region of the gene. Biochemistry 32, 6459–6464.

Palvimo, J.J., Kallio, P.J., Ikonen, T., Mehto, M., Janne, O.A., 1993. Dominant negative regulation of *trans*-activation by the rat androgen receptor: roles of the N-terminal domain and heterodimer formation. Mol. Endocrinol. 7, 1399–1407.

Pang. S., Dannull, J., Kaboo, R., Xie, Y., Tso, C.L., Michel, K., deKernion, J.B., Belldegrun, A.S., 1999. Identification of a positive regulatory element responsible for tissue-specific expression of prostate-specific antigen. Cancer Res. 57, 495–499.

Papandreou, C.N., Usmani, B., Geng, Y.P., Bogenrieder, T., Freeman, R.H., Wilk, S., Finstad, C.L., Reuter, V.E., Powell, C.T., Scheinberg, D., Magill, C., Scher, H.I., Albino, A.P., Nanus, D.M., 1998. Neutral endopeptidase 24.11 loss in metastatic human prostate cancer contributes to androgen-independent progression. Nature Med. 4, 50–57.

Rennie, P.S., Bruchovsky, N., Leco, K.J., Sheppard, P.C., McQueen, S.A., Cheng, H., Snoek, R., Hamel, A., Bock, M.E., MacDonald, B.S., et al., 1993. Characterization of two *cis*-acting DNA elements involved in the androgen regulation of the probasin gene. Mol. Endocrinol. 7, 23–36.

Riegman, P.H., Vlietstra, R.J., van der Korput, J.G.M., Brinkman, A.O., Trapman, J., 1991. The promoter of the prostate-specific antigen contains a functional androgen responsive element. Mol. Endocrinol. 5, 1921–1930.

Roche, P.J., Hoare, S.A., Parker, M.G., 1992. A consensus DNA-binding site for the androgen receptor. Mol. Endocrinol. 6, 2229–2235.

Rundlett, S.E., Miesfeld, R.L., 1995. Quantitative differences in androgen and glucocorticoid receptor DNA binding properties contribute to receptor-selective transcriptional regulation. Mol. Cell. Endocrinol. 109, 1–10.

Schena, M., Freedman, L.P., Yamamoto, K.R., 1989. Mutations in the glucocorticoid receptor zinc finger region that distinguish interdigitated DNA binding and transcriptional enhancement activities. Genes Dev. 3, 1590–1601.

Schuur, E.R., Henderson, G.A., Kmetec, L.A., Miller, J.D., Lamparski, H.G., Henderson, D.R., 1996. Prostate-specific antigen expression is regulated by an upstream enhancer. J. Biol. Chem. 271, 7043–7051.

Tan, J.A., Marschke, K.B., Ho, K.C., Perry, S.T., Wilson, E.M., French, F.S., 1992. Response elements of the androgen-regulated C3 gene [published erratum appears in J. Biol. Chem. 1992 April 15;267(11):7958]. J. Biol. Chem. 267, 4456–4466.

van der Velden, V.H., Naber, B.A., van der Spoel, P., Hoogsteden, H.C., Versnel, M.A., 1998. Cytokines and glucocorticoids modulate human bronchial epithelial cell peptidases. Cytokine 10, 55–65.

Vegeto, E., Allan, G.F., Schrader, W.R., Tsai, M.J., O'Malley, B.W., 1992. The mechanism of RU486 antagonism is dependent on the conformation of the carboxy-terminal tail of the human progesterone receptor. Cell 69, 703–712.

Zhou, Z., Corden, J.L., Brown, T.R., 1997. Identification and characterization of a novel androgen response element composed of a direct repeat. J. Biol. Chem. 272, 8227–8235.

Tumor Suppressive Effects of Neutral Endopeptidase in

Androgen-independent Prostate Cancer Cells

Jie Dai, Ruoqian Shen, Makoto Sumitomo, Jonathan S. Goldberg,

Yiping Geng, Daniel Navarro, Su Xu, Jason A. Koutcher, Mark Garzotto,

C. Thomas Powell and David M. Nanus[2]

from the Urological Oncology Research Laboratory [J.D., R.S., M.S., J.G., Y.P., D.N., D.M.N.], Department of Urology, and the Division of Hematology and Medical Oncology [D.M.N.], Department of Medicine, Joan and Stanford I. Weill Medical College of Cornell University, New York, New York 10021, the Departments of Medical Physics and Radiology [S.X., J.A.K.], and the Department of Urology [M.G., C.T.P.], Memorial Sloan-Kettering Cancer Center, New York , NY 10021.

Footnotes:

[2]To whom requests should be addressed, at New York Presbyterian Hospital-Weill Medical College, 525 E. 68th Street, Baker 1519, New York, NY 10021. Telephone: (212) 746-2920; Fax: (212) 746-6645; e-mail: dnanus@med.cornell.edu

[3]Abbreviations used: NEP, neutral endopeptidase; PC, prostate cancer

Abstract

Expression of neutral endopeptidase 24.11(NEP) is diminished in metastatic, androgen-independent prostate cancers (PC; Nature Medicine 1998;4:50). To determine the effects on androgen-independent PC cells of overexpressing cell-surface NEP, an inducible tetracycline-regulatory gene expression system was used to stably introduce and express the NEP gene in androgen-independent TSU-Pr1 cells. WT-5 cells expressed high levels of enzymatically active NEP protein when cultured in the absence of tetracycline. TN12 cells, which contain the identical vectors without the NEP gene and do not express NEP, were used as control. Expression of NEP in WT-5 cells following removal of tetracycline from the media resulted in a >80% inhibition in cell proliferation over one week (p<0.005) compared to control cells. Tumor formation occurred in the prostate glands of orthotopically injected athymic mice sacrificed at 30 days in 4/5 mice injected with 2×10^6 WT-5 cells and fed doxycyline (NEP suppressed), and in all mice injected with TN12 cells fed with or without doxycyline. In contrast, only 1 of 5 mouse prostates developed a tumor in mice injected with WT-5 cells which did not receive doxycyline. Analysis of the mechanisms of NEP-induced growth suppression revealed that NEP expression in WT-5 cells induced a 4-fold increase in the number of PC cells undergoing apoptosis, and increased expression of p21 tumor suprressor gene protein and in the level of unphosphorylated retinoblastoma protein as determined by Western blot. Flow cytometric analysis show that induced NEP expression in WT-5 cells resulted in a G1 cell cycle arrest. These data show that NEP can inhibit PC cell growth and tumorigenicity, and suggest that NEP has potential as therapy for androgen-independent prostate cancer.

3

Introduction

Neutral endopeptidase 24.11 (neprilysin, enkephalinase, CD10, EC 3.4.24.11) is a 90-110 kD cell-surface metallopeptidase which is normally expressed by numerous tissues, including prostate, kidney, intestine, endometrium, adrenal glands and lung. This enzyme cleaves peptide bonds on the amino side of hydrophobic amino acids and inactivates a variety of physiologically active peptides, including atrial natriuretic factor, substance P, bradykinin, oxytocin, Leu- and Met-enkephalins, neurotensin, bombesin, endothelin-1, and bombesin-like peptides (1-3). NEP reduces the local concentration of peptide available for receptor binding and signal transduction. The biological function of NEP appears to be organ-specific. In the central nervous system, NEP regulates enkephalin-mediated analgesia (4); in the kidney and vascular epithelium, the enzyme is involved in regulating levels of circulating atrial natriuretic factor (5); in the lung, NEP modulates tachykinins such as substance P which mediate inflammation (6); in the endometrium, NEP regulates endothelin-1 which causes vasoconstriction of endometrial arterioles during specific phases of the ovulatory cycle (7; 8). NEP has also been implicated in controlling cellular proliferation by hydrolyzing bombesin-like peptides which are potent mitogens for fibroblasts and bronchial epithelial cells (9).

Loss or decreases in NEP expression have been reported in a variety of malignancies, including renal cancer (10), invasive bladder cancer (11), poorly differentiated stomach cancer (12), small cell and non-small cell lung cancer (13), endometrial cancer (14) and prostate cancer (PC) (15). Reduced NEP may promote peptide-mediated proliferation by allowing accumulation of higher peptide concentrations

4

at the cell-surface, and facilitate the development or progression of neoplasia (16; 17).

In PCs, NEP protein is expressed in androgen-sensitive LNCaP cells, but not in androgen-independent PC cell lines (15). Furthermore, expression of NEP is transcriptionally activated by androgen in LNCaP cells and decreases with androgen-withdrawal (15; 18). Consequently, we proposed that PC cells which survive androgen-withdrawal will emerge with reduced levels of NEP. This decrease in NEP expression may result in increased growth by allowing PC cells to use neuropeptides as an alternate source to androgen to stimulate cell proliferation. To delineate the role of NEP in the development and progression of androgen-independent PC, we used a tetracycline-repressable system to introduce a full-length NEP cDNA into TSU-Pr1 cells. We report that overexpression of cell-surface NEP inhibits PC cell growth by inducing G1 cell cycle arrest, and inhibits PC cell tumorigenicity in an orthotopic model of PC.

Materials and Methods

Cell Culture. PC cell lines were maintained in RPMI 1640 media supplemented with 2 mM glutamine, 1% nonessential amino acids, 100 U/ml streptomycin and penicillin, and 10% fetal bovine serum (FBS). TSUGK27 is a TSU-Pr1 cell line stably transfected with pGK hygro and PUHD 15-1 (4.4kb) containing the coding sequence for the tet repressor adjacent to the coding sequence for the C-terminal domain of VP16 (named the tet-responsible transactivator or tTA), downstream of the hCMV promoter (19). This cell line was cultured in media containing 150 μg/ml of hygromycin and 1 μg/ml of tetracycline.

Plasmid Construction and Gene Transfer. To construct the tetracycline repressible NEP-expression vector, 1.7kb SacII-XhoI and 1.6kb XhoI-XbalI DNA fragments containing the entire wild-type NEP coding sequence, were isolated from the pClShENK vector (provided by Arris Pharmaceutical Corp., San Francisco, CA) and ligated into the pTRE vector (Clontech Laboratories, Inc.Palo Alto, CA) at the SacII and XbalI sites to generate pwtNEP. The pTRE empty vector was used as control. TSUGK27cells were co-transfected with either pwtNEP or pTRE vectors together with pSV2 which contains a neomycin resistant gene under control of the SV40 promoter using Lipofectamine following the manufacture's instructions with minor modifications (GIBCO/BRL, Gaithersburg, MD). Briefly, 2 x 10^5 cells were seeded in 6 well plates 16 hr prior to transfection. One μg of target plasmid DNA together with 0.1 μg pSV2 plasmid (ratio10:1) in serum-free MEM was mixed with 5 μl of Lipofectamine at room temperature for 30 min. Cells were then washed with serum-free MEM, incubated in the DNA-liposome mixture at 37°C for 5 hours followed by MEM containing 5% FBS for 18-24 hr.

Cells were refed with media containing 500 μg/ml of G418, 150 μg/ml hygromycin, and 1 μg/ml tetracycline for 12-15 days (GIBCO/BRL) and surviving clones were expanded to cell lines for further analysis.

Proliferation Assays. Proliferation assays were performed as previously described (20). Briefly, 10,000 cells/well were plated in duplicate 12 well tissue culture plates (Falcon Division, Becton Dickinson, Cockeysville, MD) in RPMI containing 10% FBS and counted over 3-7 days using a Coulter Counter ZM (Coulter Electronics, Hialeah, FL). All experiments were performed in triplicate on at least two separate occasions. Statistical analyses were performed using a non-paired Student's T test.

Enzyme Assays. Enzyme assays were performed as previously described (20) using Suc-Ala-Ala-Phe-pNA (Bachem Bioscience, Inc., Philadelphia, PA) as substrate. Specific activities were expressed as pmol/μg protein/minute and represent an average of two separate measurements performed in duplicate. The standard error of measurement of independent duplicate experiments was ~10 to 20% of the mean value.

Cell Cycle Analysis. Flow cytometry analysis was performed as described previously (21). Briefly, cells were rinsed twice in cold PBS, trypsinized, washed twice with PBS and fixed in cold ethanol overnight. The following day cells were resuspended in 500 μl PBS, digested with 20 μg/ml RNase at 37°C for 1 hour, chilled on ice for 10 min. and stained with propidium iodide (50 μg/ml) by incubation for 1h at room temperature in the dark. Cell cycle distribution was analyzed by flow cytometry using a Becton Dickinson FACS system.

Apoptosis Assay. Apoptotic cell number was determined by adding 2 μl of stock solution

7

containing 100 µg/ml acridine orange and 100 µg/ml ethidium bromide to a 25 µl cell suspension, and total number of cells, as well as apoptotic cells that showed shrinkage, blebing, and apoptotic bodies were counted using fluorescence microscopy. DNA fragmentation analysis was performed as previously described (22).

Protein Extraction, Immunoprecipitation and Western Blot Analysis. Protein was extracted from exponentially growing cells and analyzed by Western blotting as previously described (23) using primary antibodies 5B5 (anti-NEP; Arris Pharmaceutical Corp., San Francisco, CA.), p21, retinoblastoma, Bcl-2, p53 and cyclins A and D (Oncogene Research Products, Cambridge, MA). Blots were incubated with enhanced chemiluminescent (ECL) detection reagents (Amersham Corp., Arlington Heights, IL) and proteins detected by autoradiography by exposure of blots to Kodak XAR film for 2-15 min. Membranes were stained with 0.2% Ponceau red to assure equal protein loading and transfer. Immunoprecipitation using mAb J5 which recognizes NEP (CD10) (Beckman-Coulter Pharmaceutical, Inc., Hialeah, FL) was performed as described (24).

Orthotopic injection. Male nude mice (25-30 grams) were anesthetized with 300 µl of 5 mg/ml phentobarbital (10 µl per gram body weight) administered intraperitoneally. The abdomen was sterilely prepped with Betadine, and 1.5-cm vertical incision was made in the middle line of abdomen through the skin and peritoneum to expose the bladder and seminal vesicles. A 26-gauge needle was inserted into the parenchyma of the dorsal lobe of the prostate and advanced just below the capsule where 2×10^6 tumor cells diluted in 0.1 ml of sterile PRMI1640-10% FBS were injected. Lack of significant extra-prostatic leakage and formation of a visible bulla between prostate parenchyma and capsule were

criteria for a successful injection. The incision was closed using metal clips. Dox-Diet was obtained from Bio Serve (Frenchtown, NJ).

Nuclear magnetic resonance (NMR) imaging assay. Mice were imaged on a 4.7T 33 cm bore Bruker Omega NMR system. Images were obtained using a home built 4 turn foil solenoid radiofrequency coil tuned to 200 MHz. Image acquisition parameters included 8 slices, field of view of 25 mm, slice thickness of 2 mm, 128 x 128, matrix (200u in plane resolution), repetition interval (TR) of 500 ms, and echo time (TE) of 16 ms.

RESULTS

Expression of NEP in TSU-Pr1 cells. TSUGK27 cells, a derivative of TSU-Pr1 cells which contains the tetracycline regulator and a hygromycin resistance plasmid (19), do not express NEP protein or exhibit NEP enzyme activity. TSUGK27 cells were transfected with pwtNEP (or pTRE control vector) together with a neomycin resistance vector, and antibiotic resistant stable clones screened by measuring NEP catalytic activity in cells cultured in the presence or absence of 1 µg/ml of tetracycline (Figure 1A). Removal of tetracycline from the media resulted in a significant increase in NEP enzyme activity in at least four clones (WT5, WT6, WT12 and WT24). TSUGK27 cells containing the pTRE empty vector without the NEP gene (TN cells) did not exhibit enzyme activity. Integration of pTRE into genomic DNA of TN cell lines was verified by Southern blotting with a probe specific for pTRE vector sequence (not shown). Western blot of NEP protein immunoprecipitated with mAb J5 which recognizes NEP showed the presence of NEP protein in WT5 and WT24 cells following the removal of tetracycline from the media similar to that observed in LNCaP cells which constitutively express NEP but not in control TN12 or TN15 cells (Figure 1B). Flow cytometry confirmed the expression of cell-surface NEP in WT cell lines (data not shown). WT5 cells were further characterized for NEP enzyme inducibility. NEP enzyme activity was suppressed when WT-5 cells were cultured in 1 µg/ml of tetracycline, and enzyme activity increased with decreasing concentrations of tetracycline ranging from 1000 to 0 ng/ml (Figure 1C). Enzyme activity reached maximum levels at 72-96 hrs after the removal of tetracycline from the media (Figure 1D). Thus, induction of NEP enzyme activity was both time-course-dependent and

10

dose-dependent.

NEP expression induces growth inhibition in vitro and inhibits tumorigenicity in athymic mice. WT5 and control TN12 cells were cultured in the presence or absence of 1 μg/ml of tetracycline over one week. WT5 cells cultured without tetracycline were > 80% growth inhibited compared to WT5 cells cultured with tetracycline or control cells (p < 0.005; Figure 2A). To determine whether NEP expression could inhibit tumorigenicity in vivo, we used an orthotopic model of PC. WT5 and TN12 cells were injected directly into the prostate gland of athymic mice. One half of the animals received doxycyline in their feed, and all animals were sacrificed at 30 days. Magnetic resonance imaging was performed on one animal from each treatment group prior to sacrifice. As illustrated in Figure 2B, tumors were detected in the prostate of two animals injected with TN12 cells regardless of whether they received tetracycline, and in the prostate of one animal fed with tetracycline (NEP expression suppressed) which was injected with WT5 cells. However, no tumor was detected in the animal injected with WT5 cells which did not receive tetracycline (NEP expressed). Autopsies of all animals revealed 100% tumor formation in animals receiving TN12 cells, and in 4 of 5 (80%) animals injected with WT5 cells and fed with tetracycline. Only 1 of 5 animals injected with WT5 cells which did not receive tetracycline developed a tumor, which was appreciably smaller than other tumors formed (Table 1).

NEP expression induces apoptosis, G1 cell cycle arrest, and expression of p21 protein. Analysis of WT5 cells following removal of tetracycline from the media for 7 days revealed nuclear fragmentation and chromatin condensation, consistent with cells

11

undergoing apoptosis (Figure 3A), which was confirmed by gel electrophoresis showing nucleasomal fragments in the DNA derived from WT5 cells cultured without tetracycline (Figure 3B). As shown by flow cytometric analysis, removal of tetracycline for 7 days induced apoptosis in 17.8% of WT-5 cells. This apoptosis was not observed in TN12 cells or in WT5 cells cultured in tetracycline, which suppresses NEP expression. Cell cycle analysis of TN12 cells before and after the removal of tetracycline from the media revealed no differences in the distribution of cells within phases of the cell cycle (Figure 3C). Similar to TN12 cells, 54.7% of WT-5 cells cultured in media containing tetracycline (which suppresses NEP expression) were in the G1 phase of the cell cycle. However, induction of NEP expression following removal of tetracycline from the media resulted in G1 arrest, with 73.9% of WT-5 cells in G1 phase. Analysis of cell cycle proteins which may contribute to an arrest in G1 revealed a 3-4 fold increase in p21 protein levels, and a reduction of phosphorylated retinoblastoma protein (ppRb) to the unphosphorylated form in WT5 cells cultured without tetracycline (Figure 3D). In contrast, no alterations in the expression pattern of cyclin A, cyclin D, p27 or p53 were observed.

Discussion

NEP inactivates neurotensin, bombesin and endothelin-1, all of which have been implicated in progression to androgen-independent PC (25-27). We previously reported that NEP expression is decreased in androgen-independent PC cell lines in vitro, and in tumor cells of metastatic biopsy specimens in vivo from patients with androgen-independent PC (15). Expression of NEP is transcriptionally activated by androgen in androgen-dependent PC cells and decreases with androgen-withdrawal (15). Consequently, PC cells which survive androgen-withdrawal can emerge with reduced levels of NEP. This decrease in NEP expression can contribute to the development of androgen-independent PC by allowing PC cells to use neuropeptides as an alternate source to androgen to stimulate cell proliferation.

The current study was aimed at further delineating the anti-tumor effects of NEP and its potential use for inhibiting androgen-independent PC cell growth. In this regard, we had previously shown that androgen-induced growth repression of androgen-independent PC3 cells expressing androgen receptor (PC3/AR) and of an androgen-independent subline of LNCaP cells results in part from androgen-induced expression of NEP in these cells (20). However, the growth inhibition observed was modest (~20-30%) and the level of NEP specific enzyme activity achieved following androgen-stimulation was relatively low. Recombinant NEP also inhibits androgen-independent PC cell growth, but it is difficult to obtain sustained serum levels of NEP in mice using recombinant NEP (13), and we did not observe significant inhibition of tumorigenicity of PC xenografts in athymic mice receiving intraperitoneal recombinant NEP daily for 30 days (C.N.

Papandreou and D.M. Nanus, unpublished data). Therefore, we constructed an inducible system of NEP expression to more fully examine the effects of expressing cell-surface NEP in androgen-independent PC cells.

Our data show that NEP can regulate androgen-independent PC cell proliferation. Moreover, expression of NEP inhibits xenograft tumor formation in the prostate gland of athymic mice, suggesting NEP can function as a tumor suppressor of prostate cancer. The mechanism of NEP induced growth inhibition presumably involves the inactivation of its neuropeptide substrates, such as bombesin, endothein-1 and neurotensin, each of which has been implicated in androgen-independent PC growth (28). We observed that overexpression of NEP resulted in an increase in cells undergoing apoptosis. Recent studies suggest that bombesin and endothelin-1 can act as survival factors, and inhibit apoptosis (29-31). Thus, expression of NEP which leads to inactivation of these neuopeptides would allow for greater numbers of cells to undergo cell death. In addition, overexpression of cell-surface NEP led to G1 cell cycle arrest, presumably mediated in part by the induction of p21 protein expression and dephosphorylation of Rb. We recently have shown that overexpression of NEP in WT-5 cells inhibits neuropeptide-mediated phosphorylation of focal adhesion kinase (p125FAK) (24). Zhao, et al, reported that overexpression of a dominant-negative FAK mutant inhibited cell cycle progression at G1, and induced p21 expression (32), leading to the possibility that NEP's induction of growth arrest results in part from inhibition of FAK phosphorylation.

LNCaP cells constitutively express NEP and exhibit some of the characteristics observed in WT-5 cells expressing NEP, including a longer doubling time (33), diminished

14

ability to migrate in extracellular matrix (24; 34) and decreased tumorigenicity in athymic mice (34; 35), compared to androgen-independent PC cells which lack NEP expression. In addition, inhibition of NEP enzyme activity in LNCaP cells results in an increase in FAK phosphorylation (24). However, the complete phenotype observed in WT-5 cells induced to express NEP is not present in LNCaP cells. This may result from the lower levels of NEP protein and enzyme activity in LNCaP cells compared to WT-5 cells, or from lack of downstream mediators of NEP action which are present in TSU-Pr1 cells but not in LNCaP cells.

In summary, the current study emphasizes the consequences of decreased NEP expression in PC cells by studying the effects of re-expressing cell-surface NEP. These experiments suggest overexpression of NEP in PC cells results in multiple effects including growth inhibition, induction of apoptosis, cell cycle arrest and the inhibition of tumor formation. Augmentation of NEP expression by delivery of exogenous cell-surface NEP using gene constructs is a potential approach to the treatment of hormone refractory PC.

Figure 1. Analysis of TSUGK27 cells stably transfected with pwtNEP. A) TSUGK27 cells stably transfected with pwtNEP were cultured in the presence or absence of 1 μg/ml tetracycline (tet) for 48-72 hr, and NEP specific enzyme activity determined. Data shown represent the average of 1-3 independent experiments for each clone. Error bars indicate standard errors. B) Cells cultured in RPMI containing FCS and no tetracycline were assessed for NEP expression. Identical numbers of cells were lysed and immunoprecipitated with mAb J5, separated by SDS-PAGE, transferred to nitrocellulose, and probed with rabbit polyclonal Ab 5B5 which recognizes NEP. LNCaP cells, which constitutively express NEP were used as control. C) Enzyme activities of WT5 cells were determined following incubation in media containing various concentrations of tetracycline, and D) at various time points following removal of 1 μg/ml of tetracyline from the media. Representative data are shown from one experiment performed in duplicate on at least 2 separate occasions. Data are expressed as relative NEP activity with 100% equal to the average enzyme activity in cells cultured without tetracycline (panel C), or enzyme activity in cells 96 hrs after removal of tetracycline (panel D). Error bars indicate standard error.

Figure 2. A. NEP expression induces growth inhibition in vitro. WT5 and TN12 were cultured for 7 days with and without tetracycline and cell number determined. Data representative of one experiment performed in triplicate. Error bars indicate standard.

B. Nuclear magnetic resonance (NMR) imaging of mouse prostates injected with PC cells. High spatial resolution 1H NMR images of the pelvis were obtained of mice

injected orthotopically into the prostate with 1 x 10^6 cells of TN12 or WT5 cells. Mice were fed with regular feed or Dox-Diet (feed containing doxycycline), beginning 3 days prior to orthotopic injection. All mice were sacrificed 30 days after implantation. Imaging was performed prior to sacrifice in randomly selected animals. The images in the upper two panels represent images of prostate glands injected with TN12 control cells and fed with Dox-Diet (upper left) or regular feed without doxycycline (upper right). Note tumor formation in both animals. The images in the lower two panels represent images of prostate glands injected with WT-5 cells and fed with Dox-Diet (lower left) or regular feed without doxycycline (lower right). Note absence of tumor formation in mice not receiving doxycyline (NEP expressed). T = tumor, B = bladder, P = prostate.

Figure 3. NEP expression induces apoptosis and cell cycle G1 arrest. A. Photomicrograph of WT5 cells stained with acridine orange and ethidium bromide cultured in the presence (left) or absence of tetracycline (right). Note apoptotic bodies in cells (arrow) in which NEP expression is induced by removing tetracycline. B. DNA fragmentation gel electrophoresis of WT5 cells cultured in the presence of absence of tetracycline for 3, 5 and 7 days. Note DNA laddering in cells cultured without tetracycline (NEP expressed) for 7 days. C. Flow cytometric analysis of TN-12 and WT5 cells cultured in the presence of absence of tetracycline for 7 days. Note there is no significant difference in cell cycle histograms between TN-12 cultured with tetracycline (upper left) or without tetracycline (upper right), or with WT-5 cultured in tetracycline (lower left). Following removal of tetracycline from WT5 for 7 days, increase in cells in G1 and percent apoptotic

cells occurs (lower right). D. Cell lysates from WT5 cells cultured in the presence of absence of tetracycline for 3, 5 and 7 days were separated on an SDS-PAGE, transferred to nitrocellulose and probed with mAbs to the proteins indicated. Note increase in p21 protein and decrease in the level of phosphorylated retinoblastoma (Rb) protein. Similar results obtained on at least one other occasion using different cell lysates.

Table 1. NEP Inhibits Xenograft Tumor Formation in the Prostates of Athymic Mice.

Cell Line	Dox	Tumor Formation	Tumor Size $(mm^3)^1$	Mouse Weight (gm)
WT-5	+	4/5 (80%)	0.43 (0.11)	6.1 (2.4)
WT-5	-	1/5(20%)	0.07 (0.07)	5.7 (2.8)
TN-12	+	4/4 (100.0%)	0.91 (0.35)	5.5 (2.3)
TN-12	-	4/4 (100.0%)	1.04 (0.42)	6.2 (1.9)

Athymic mice were injected with 2×10^6 cells as described in Material and Methods. Animal were fed with Dox-diet or regular feed and sacrificed at 30 days.

[1] Tumor size measured in mm^3 and calculated as length x width x depth/2. Tumor measurements were performed by two researchers independently. Animal weight measured in grams; ( )= standard of error.

References

1. Checler, F., Emson, P.C., Vincent, J.P., and Kitabgi, P. Inactivation of neurotensin by rat brain synaptic membranes. Cleavage at the Pro10-Tyr11 bond by endopeptidase 24.11 (enkephalinase) and a peptidase different from proline- endopeptidase. J.Neurochem., *43*: 1295-1301, 1984.

2. Kenny, A.J. Endopeptidase-24.11: putative substrates and possible roles. Biochem Soc.Trans., *21*: 663-668, 1993.

3. Shipp, M.A., Tarr, G.E., Chen, C.Y., Switzer, S.N., Hersh, L.B., Stein, H., Sunday, ME, and Reinherz, E.L. CD10/neutral endopeptidase 24.11 hydrolyzes bombesin-like peptides and regulates the growth of small cell carcinomas of the lung. Proc Natl Acad Sci USA, *88*: 10662-10666, 1991.

4. Roques, B.P., Fournie-Zaluski, M.C., Soroca, E., Lecomte, J.M., Malfroy, B., Llorens, C., and Schwartz, J.C. The enkephalinase inhibitor thiorphan shows antinociceptive activity in mice. Nature, *288*: 286-288, 1980.

5. Soleilhac, J.M., Lucas, E., Beaumont, A., Turcaud, S., Michel, J.B., Ficheux, D., Fournie-Zaluski, M.C., and Roques, B.P. A 94-kDa protein, identified as neutral endopeptidase-24.11, can inactivate atrial natriuretic peptide in the vascular endothelium. Mol.Pharmacol., *41*: 609-614, 1992.

6. Sunday, M.E., Hua, J., Torday, J.S., Reyer, B., and Shipp, M.A. CD/10 neutral endopeptidase 24.11 in developing human fetal lung. J.Clin.Invest., *90*: 2517-2525, 1992.

7. Pekonen, F., Nyman, T., and Rutanen, E.M. Differential expression of mRNAs for endothelin-related proteins in human endometrium, myometrium and leiomyoma. Mol Cell Endocrinol., *103*: 165-170, 1994.

8. Casey, M.L., Smith, J.W., Nagai, K., Hersh, L.B., and MacDonald, P.C. Progesterone-regulated cyclic modulation of membrane metalloendopeptidase (enkephalinase) in human endometrium. J Biol Chem, *34*: 23041-23047, 1991.

9. King, K.A., Hua, J., Torday, J.S., Drazen, J.M., Graham, S.A., Shipp, M.A., Sunday, and ME. CD10/neutral endopeptidase 24.11 regulates fetal lung growth and maturation in utero by potentiating endogenous bombesin-like peptides [see comments]. J Clin Invest., *91*: 1969-1973, 1993.

10. Gohring, B., Holzhausen, H.J., Meye, A., Heynemann, H., Rebmann, U., Langner, J., and Riemann, D. Endopeptidase 24.11/CD10 is down-regulated in renal cell cancer. Int J Mol Med, *2*: 409-414, 1998.

11. Koiso, K., Akaza, H., Ohtani, M., Miyanaga, N., and Aoyagi, K. A new tumor marker for bladder cancer. Int J Urol, *1*: 33-36, 1994.

12. Sato, Y., Itoh, F., Hinoda, Y., Ohe, Y., Nakagawa, N., Ueda, R., Yachi, A., and Imai, K. Expression of CD10/neutral endopeptidase in normal and malignant tissues of the human stomach and colon. J Gastroenterol, *31*: 12-17, 1996.

13. Cohen, A.J., Bunn, P.A., Franklin, W., Magillsolc, C., Hartmann, C., Helfrich, B., Gilman, L., Folkvord, J., Helm, K., and Miller, Y.E. Neutral endopeptidase variable expression in human lung, inactivation in lung cancer, and modulation of peptide induced calcium flux. Cancer Res., *56*: 831-839, 1996.

14. Pekonen, F., Nyman, T., Ammala, M., and Rutanen, E.M. Decreased expression of messenger RNAs encoding endothelin receptors and neutral endopeptidase 24.11 in endometrial cancer. Br.J Cancer, *71*: 59-63, 1995.

15. Papandreou, C.N., Usmani, B., Geng, Y.P., Bogenrieder, T., Freeman, R.H., Wilk, S., Finstad, C.L., Reuter, V.E., Powell, C.T., Scheinberg, D., Magill, C., Scher, H.I., Albino, A.P., and Nanus, D.M. Neutral endopeptidase 24.11 loss in metastatic human prostate cancer contributes to androgen-independent progression. Nature Med, *4*: 50-57, 1998.

16. Shipp, M.A. and Look, A.T. Hematopoietic differentiation antigens that are membrane-associated enzymes: Cutting is the key! Blood, *84*: 1052-1070, 1993.

17. Nanus, D.M., Papandreou, C.N., and Albino, A.P. Expression of cell-surface peptidases in neoplastic cells. In: J. Kenny and C.M. Boustead (eds.), Cell-surface peptidases in health and disease, pp. 353-369, Oxford: BIOS Scientific Publishers. 1997.

18. Shen, R., Sumitomo, M., Dai, J., Hardy, D.O., Navarro.D., Usmani, B., Papandreou, C., Hersh, L.B., Shipp, M.A., Freedman, L.P., and Nanus, D.M. Identification and characterization of two androgen response regions in the human neutral endopeptidase gene. Mol Cell Endocrinol., *in press*: 2000.

19. Gschwend, J.E., Fair, W.R., and Powell, C.T. Evaluation of the tetracycline-repressible transactivator system for inducible gene expression in human prostate cancer cell lines. Prostate, *33*: 166-176, 1997.

20. Shen, R., Sumitomo, M., Dai, J., Harris, A., Kaminetzky, D., Gao, M., Burnstein, K., and Nanus, D.M. Androgen-induced growth inhibition of androgen receptor expressing androgen-independent prostate cancer cells is mediated by increased levels of neutral endopeptidase. Endocrinology, *141*: 1699-1704, 2000.

21. Gao, M., Ossowski, L., and Ferrari, A.C. Activation of Rb and decline in androgen receptor protein precede retinoic acid-induced apoptosis in androgen-dependent LNCaP

cells and their androgen-independent derivative. Journal of Cellular Physiology, *179*: 336-346, 1999.

22. Dai, J., Weinberg, R.S., Waxman, S., and Jing, Y. Malignant cells can be sensitized to undergo growth inhibition and apoptosis by arsenic trioxide through modulation of the glutathione redox system. Blood, *93*: 268-277, 1999.

23. Papandreou, C.N., Bogenrieder, T., Loganzo, F., Chao, M.V., Nanus, D.M., and Albino, A.P. Mutation and expression of the low affinity neurotropin receptor in human malignant melanoma. Melanoma Res., *6*: 373-378, 1996.

24. Sumitomo, M., Shen, R., Dai, J., Geng Y., Navarro, D., Boileau, G., Papandreou, C., Giancotti, F.G., Knudsen, B., and Nanus, D.M. Neutral endopeptidase inhibits prostate cancer cell migration by blocking focal adhesion kinase (FAK) signaling. J Clin Invest., *submitted*: 2000.

25. Di Sant'Agnese, P.A. Neuroendocrine differentiation in prostatic carcinoma. Cancer, *75*: 1850-1859, 1995.

26. Abrahamsson, P.A. Neuroendocrine differentiation and hormone-refractory prostate cancer. [Review]. Prostate - Supplement, *6*: 3-8, 1996.

27. Cussenot, O., Villette, J.M., Valeri, A., Cariou, G., Desgrandchamps, F., Cortesse, A., Meria, P., Teillac, P., Fiet, J., and Le Duc, A. Plasma neuroendocrine markers in patients with benign prostatic hyperplasia and prostatic carcinoma. J Urol, *155*: 1340-1343, 1996.

28. Nelson, J.B. and Carducci, M.A. Small bioactive peptides and cell surface peptidases in androgen-independent prostate cancer. Cancer Invest., *18*: 87-96, 2000.

29. Kresch, M., Christian, C., Zhu, L., Obe, M., Sanders, M.M., and Hussain, N. Bombesin inhibits apoptosis in developing fetal rat. Lung, *177*: 241-251, 1999.

30. Shichiri, M., Yokokura, M., Marumo, F., and Hirata, Y. Endothelin-1 inhibits apoptosis of vascular smooth muscle cells induced by nitric oxide and serum deprivation via MAP kinase pathway. Arterioscler Thromb Vasc Biol, *20*: 989-997, 2000.

31. Eberl, L.P., Valdenaire, O., Saintgiorgio, V., Jeannin, J.F., and Juillerat-Jeanneret, L. Endothelin receptor blockade potentiates FasL-induced apoptosis in rat colon carcinoma cells. Int J Cancer, *86*: 182-187, 2000.

32. Zhao, J.H., Reiske, H., and Guan, J.L. Regulation of the cell cycle by focal adhesion kinase. J Cell Biol, *143*: 1997-2008, 1998.

33. Peehl, D.M. The male reproductive system: Prostatic cell lines. In: R.J. Hay, J.G. Park and A. Gazdar (eds.), Atlas of human tumor cell lines, pp. 387-411, San Diego: Academic Press, Inc. 1994.

34. Passaniti, A., Isaacs, J.T., Haney, J.A., Adler, S.W., Cujdik, T.J., Long, P.V., and Kleinman, H.K. Stimulation of human prostatic carcinoma tumor growth in athymic mice and control of migration in culture by extracellular matrix. Int J Cancer, *51*: 318-324, 1992.

35. Thalmann, G.N., Anezinis, P.E., Chang, S.M., Zhau, H.E., Kim, E.E., Hopwood, V.L., Pathak, S., von Eschenbach, A.C., and Chung, L.W. Androgen-independent cancer progression and bone metastasis in the LNCaP model of human prostate cancer. Cancer Res., *54*: 2577-2581, 1995.
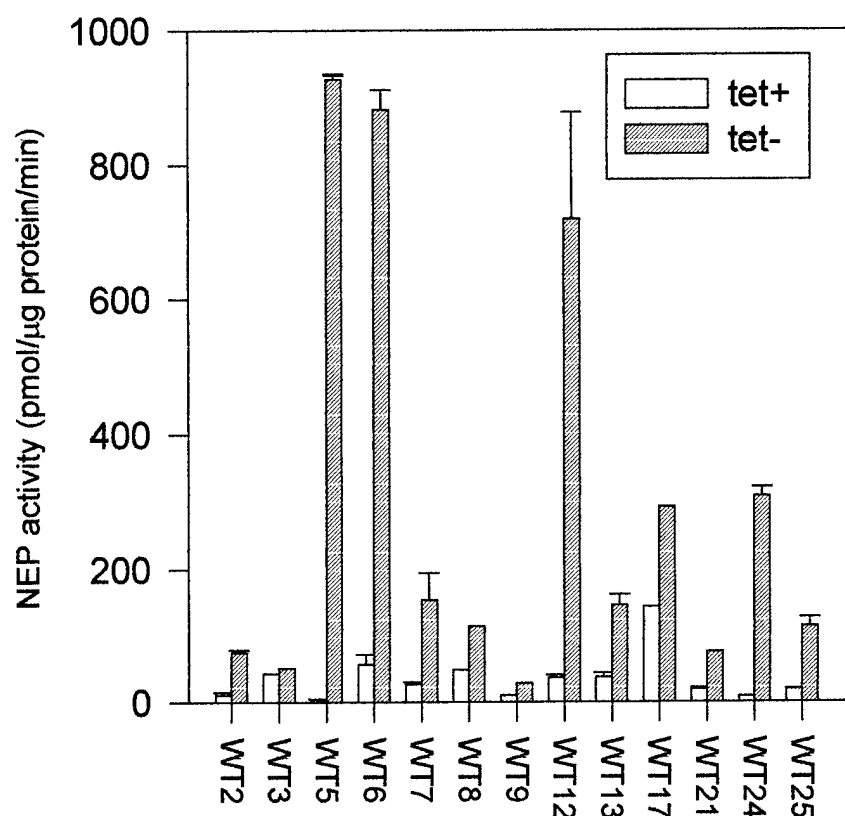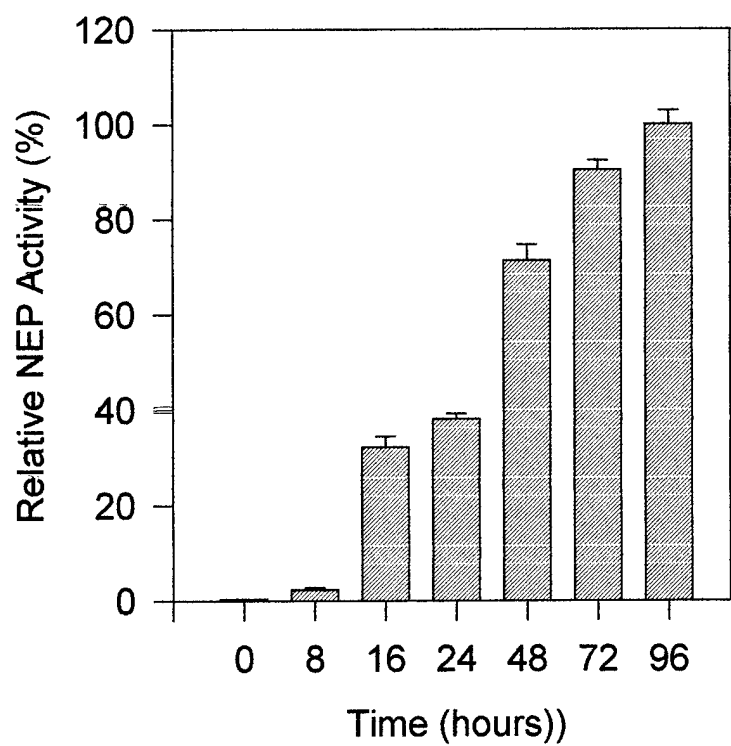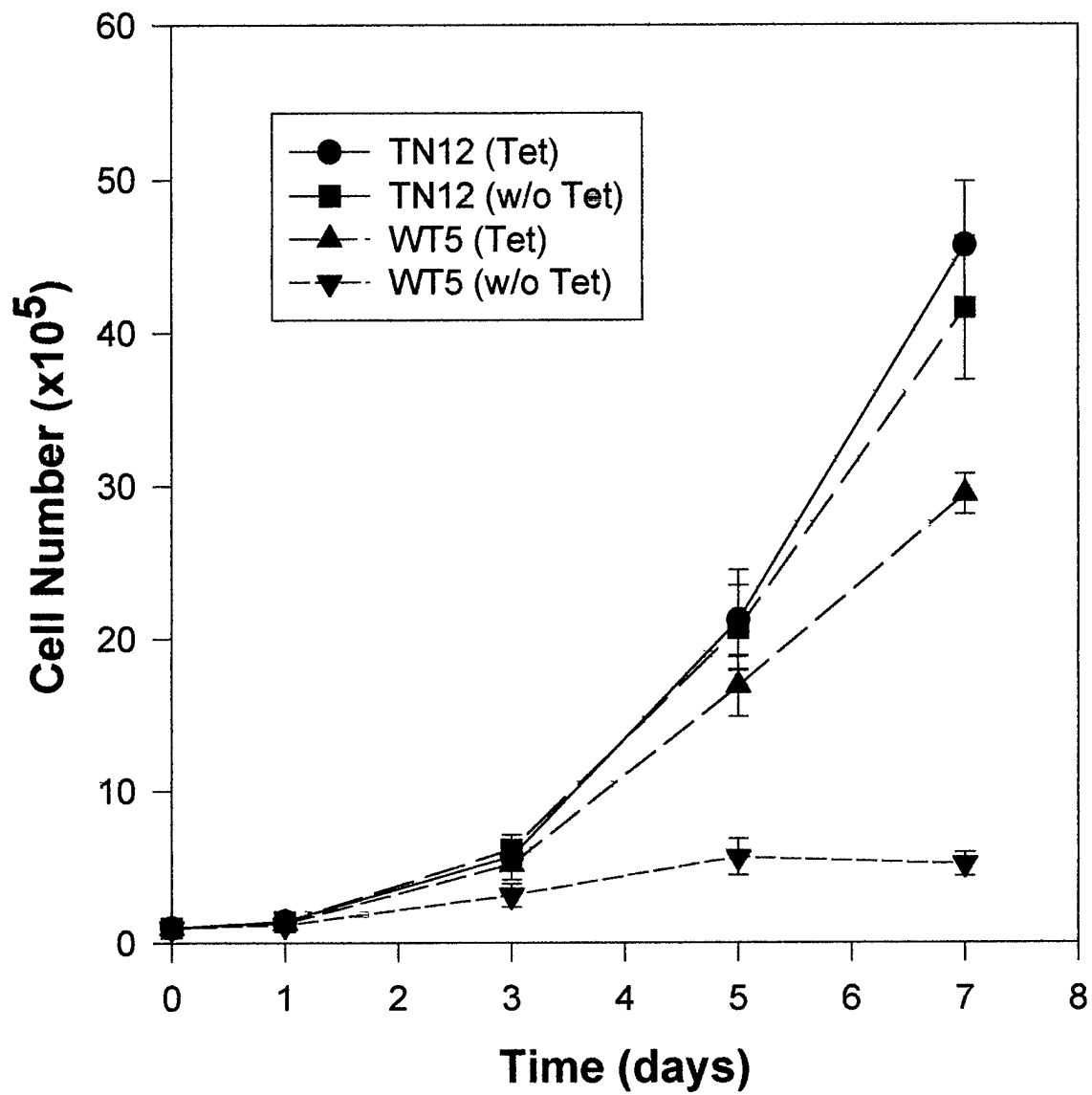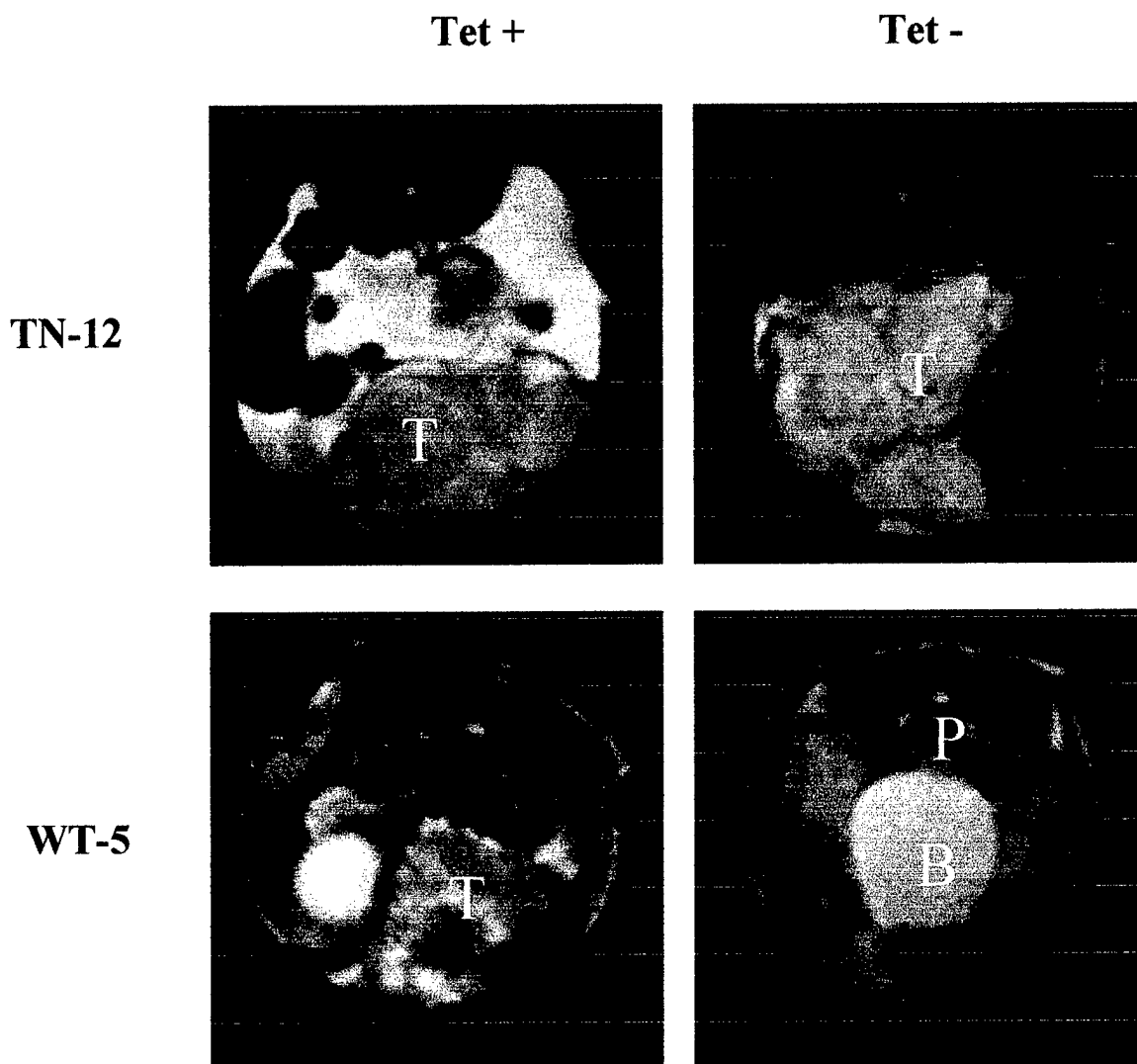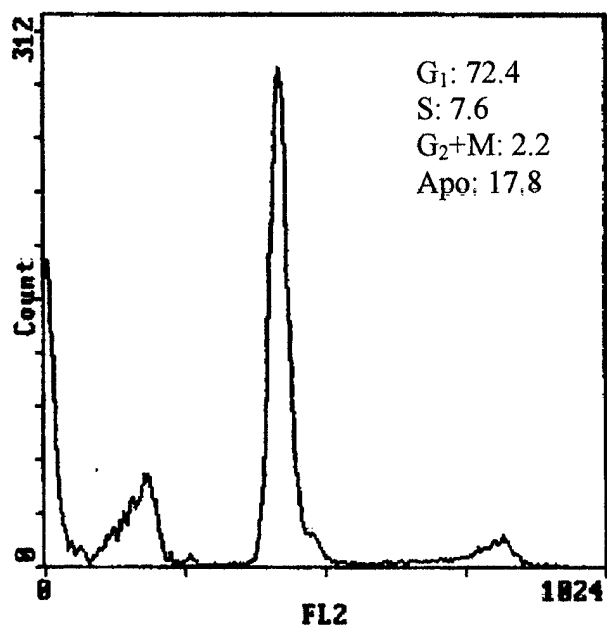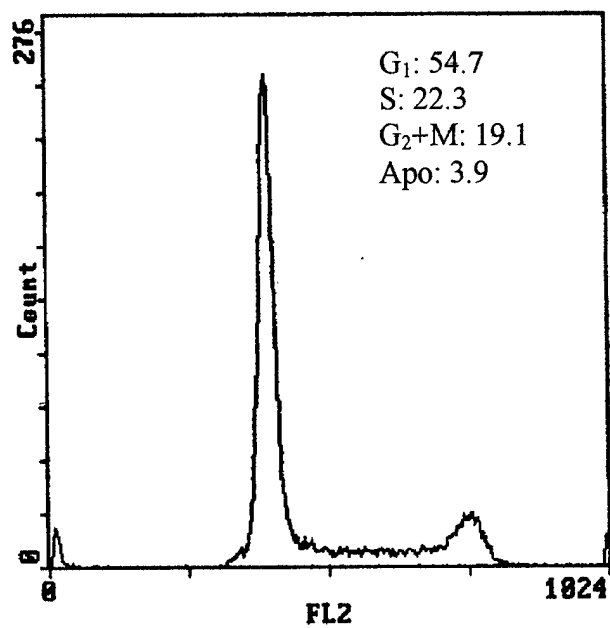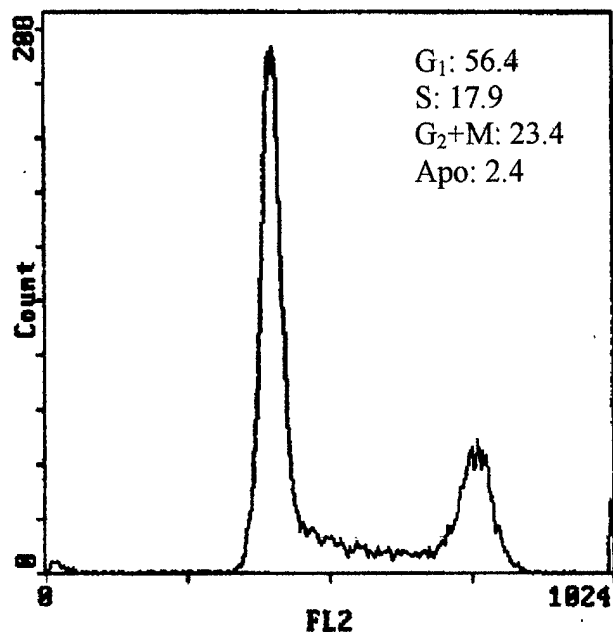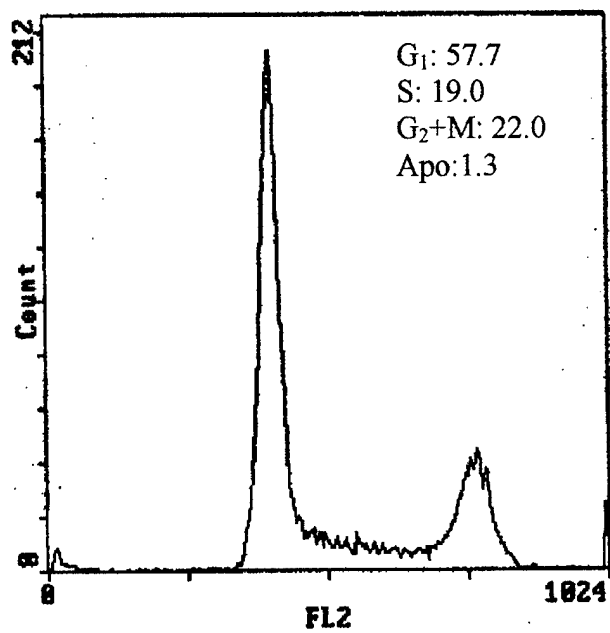
Tet + **Tet -**
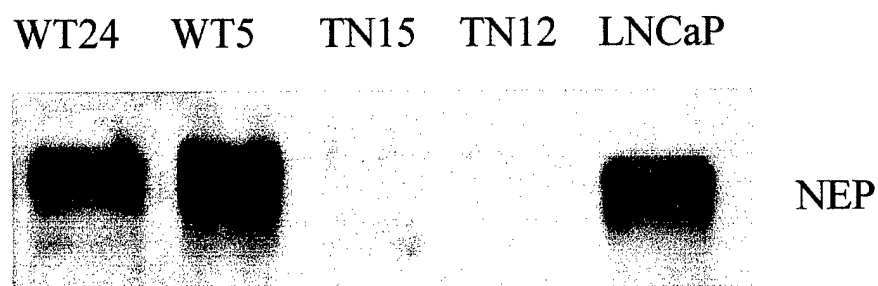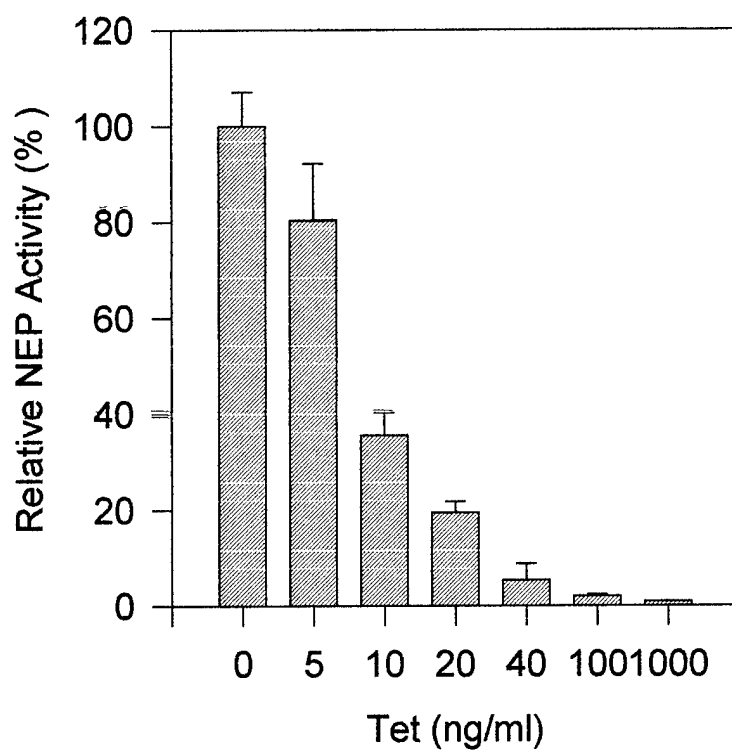
TN-12

WT-5

FIGURE 3A

Neutral Endopeptidase Inhibits Prostate Cancer Cell Migration by Blocking Focal Adhesion Kinase (FAK) Signaling.

By Makoto Sumitomo[1], Ruoqian Shen[1], Marc Walburg[3], Jie Dai[1], Yiping Geng[1], Daniel Navarro[1], Guy Boileau[4], Christos N. Papandreou[5], Filippo G. Giancotti[6], Beatrice Knudsen[3] and David M. Nanus[1,2]

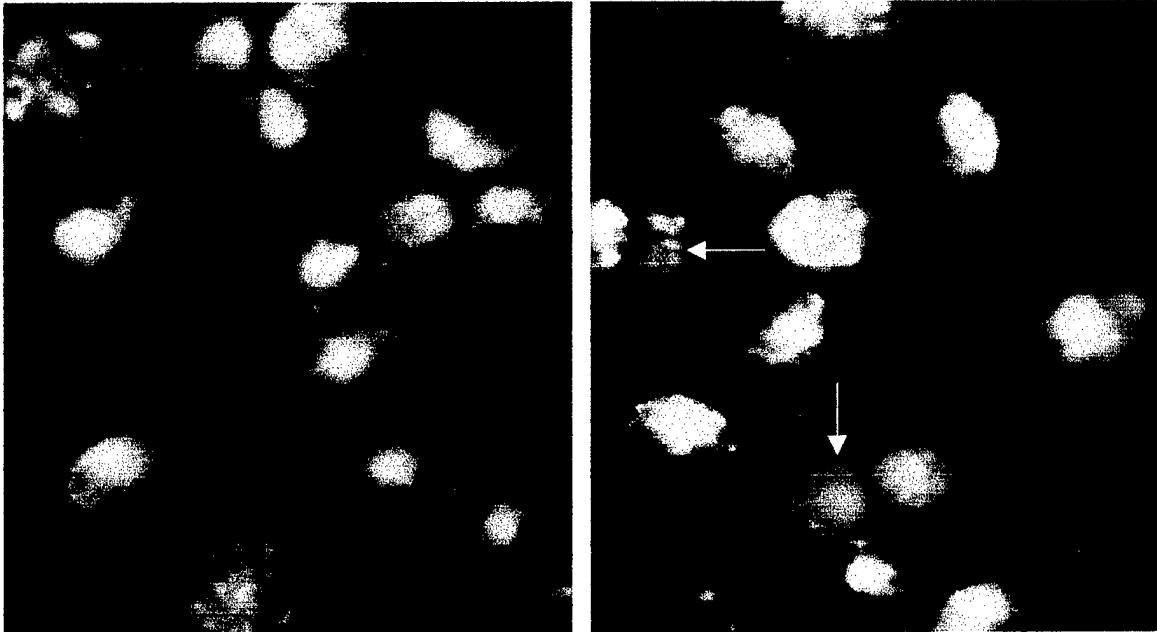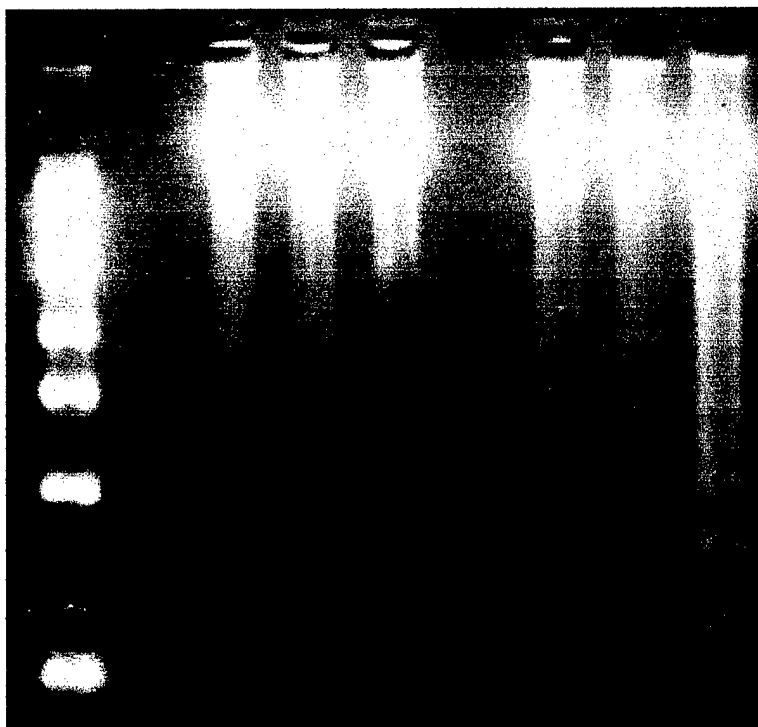From the [1]Urologic Oncology Research Laboratory, Department of Urology, [2]the Division of Hematology and Medical Oncology, Department of Medicine, and [3]the Department of Pathology, Weill Medical College of Cornell University, New York, New York 10021, [4]the Department of Biochemistry, Faculty of Medicine, University of Montreal, Montreal, Quebec, H3C 3J7, Canada, [5]the Department of Genitourinary Medical Oncology, University of Texas-M.D. Anderson Cancer Center, Houston, Texas 77030, and [6]the Cellular Biochemistry and Biophysics Program, Sloan-Kettering Institute for Cancer Research, New York, New York 10021.

Correspondence: Dr. David M. Nanus

Weill Medical College of Cornell University, 525 E. 69th Street, New York, NY 10021

Telephone: (212) 746-2920; Fax: (212) 746-6645; e-mail: dnanus@med.cornell.edu

Abstract

Neutral endopeptidase 24.11 (NEP, CD10) is a cell-surface enzyme expressed by prostatic epithelial cells that cleaves and inactivates neuropeptides implicated in the growth of androgen-independent prostate cancer (PC). NEP substrates such as bombesin and endothelin-1 induce cell migration. We investigated the mechanisms of NEP regulation of cell migration in PC cells, including regulation of phosphorylation on tyrosine of focal adhesion kinase (FAK). Western analyses and cell migration assays revealed an inverse correlation between NEP expression and the levels of FAK phosphorylation and cell migration in PC cell lines. Constitutively expressed NEP, recombinant NEP, and induced NEP expression using a tetracycline-repressive expression system inhibited bombesin and endothelin-1 stimulated FAK phosphorylation and cell migration. This results from NEP-induced inhibition of neuropeptide-stimulated association of FAK with cSrc protein. Expression of a mutated catalytically inactive NEP protein also resulted in partial inhibition of FAK phosphorylation and cell migration. Co-immunoprecipitation experiments show that NEP associates with tyrosine-phosphorylated Lyn kinase, which then binds the p85 subunit of phosphatidylinositol 3-kinase (PI3-K) resulting in NEP-Lyn-PI3-K protein complex. This complex competitively blocks FAK-PI3-K interaction, suggesting that the NEP protein inhibits cell migration via a protein-protein interaction independent of its catalytic function. These experiments demonstrate that NEP can inhibit FAK phosphorylation on tyrosine and PC cell migration through multiple pathways, and suggest that NEP plays a crucial role in regulating cell migration which contributes to invasion and metastases in PC cells.

Key Words: bombesin, cell-surface peptidase, invasion, Lyn kinase, PI3-kinase

Introduction

The molecular events involved in prostate cancer (PC) development and progression are not well defined. Recent data implicate neuropeptides such as bombesin. endothelin-1 (ET-1) and neurotensin in various stages of PC, including PC development, PC cell migration and progression to hormone-independence (1-3). Access of these neuropeptides to their cell-surface receptors is negatively regulated in part by neutral endopeptidase 24.11 (NEP, CD10), a 90-110 kDa zinc-dependent metallopeptidase which cleaves peptide bonds on the amino side of hydrophobic amino acids. The catalytic domain of NEP is located in the extracellular part of the NEP protein. NEP, which is expressed on benign prostate epithelial cells, normally functions to reduce local concentrations of neuropeptide available for receptor binding and signal transduction (4, 5). A decrease in NEP expression in PC cells may contribute to PC tumor progression by allowing neuropeptides to bind their receptors and provide growth stimulatory pathways (6).

NEP has been implicated in the inhibition of cell migration in neutrophils and lung fibroblasts (7, 8). In PC cells, NEP is highly expressed by LNCaP cells (6), a PC cell line which is often used as a model for hormone-sensitive PC. LNCaP cells do not migrate or invade though ECM, in contrast to hormone-insensitive PC cell lines TSU-Pr1, DU-145 or PC-3 which do not express NEP (6) and exhibit cell migration which can be increased by the NEP-substrate bombesin (2). This correlation in PC cells of loss of NEP expression and cell migration could be explained by the catalytic capability of NEP to inactivate neuropeptides such as bombesin which promote cell migration.

The biological and regulatory effects of NEP are presumed only to result from its enzymatic function (4, 5). However, recent data suggest that NEP may possess other biological properties

3

in addition to its ability to catalytically inactivate neuropeptide substrates and that NEP protein expression in of itself can effect signal transduction pathways which regulate cell growth (9, 10) and apoptosis (11). In the present study, we used LNCaP cells and hormone-independent TSU-Pr1 PC cells to investigate the mechanisms of NEP action by assessing the effects of NEP on regulating phosphorylation of focal adhesion kinase (FAK) on tyrosine and cell migration.

Methods

Cell Culture and Reagents. PC cell lines were maintained in RPMI 1640 media supplemented with 2 mM glutamine, 1% nonessential amino acids, 100 U/ml streptomycin and penicillin, and 10% fetal calf serum (FCS). Reagents used include recombinant NEP (rNEP; Arris Pharmaceutical, Inc., S. San Francisco, CA); CGS24592 (Novartis Pharmaceutical, Summit, NJ) (12); and PP2 (Calbiochem-Novabiochem Ltd., La Jolla, CA). The following antibodies (Ab) were used: mouse monoclonals to NEP (J5, Beckman Coulter, Fullerton, CA); to Lyn (H-6), to cSrc (B-12), to anti-pTyr (PY20; Santa-Cruz Biotechnology Inc., Santa Cruz, CA); rabbit polyclonals to NEP (5B5, Arris Pharmaceutical Corp.), to p85 (Upstate Biotechnology, Lake Placid, NY), to FAK (C-20), to Lyn (clone 44), and to cSrc (SRC-2; Santa-Cruz Biotechnology).

Plasmid Construction and Gene Transfer. TSU-GK27-NEP (WT-5) and TSU-GK27-Neo (TN-12) cells were constructed and maintained as previously described (6). A mutated NEP gene containing a substitution of Val for the catalytic Glu residue was constructed by PCR-based site-directed mutagenesis (13). A 1525 bp PCR DNA fragment was cloned and sequenced entirely to confirm the presence of the mutation and the absence of other bp changes. A 1339 bp DNA fragment containing the mutated region was used to replace the equivalent fragment in the expression vector pSVCALLA, which when transfected into COS-1 cells promoted the expression of high amounts of human NEP but no enzymatic activity, as previously reported for the same mutation in rabbit NEP (14). A DNA fragment containing the mutated sequence was used to replace the equivalent fragment in the pTRE-NEP vector. The pTRE-mutated NEP vector was then introduced into TSU-GK27 cells and stable cell lines expressing an inducible mutated NEP protein isolated (M-22 cells) as described (6).

Enzyme Assays. Cells were incubated with or without tetracycline and NEP specific enzyme activity were performed on total cell lysates as previously described (15) using Suc-Ala-Ala-Phe-pNA (Bachem Bioscience, Inc., Philadelphia, PA) as substrate. Specific activities were expressed as pmol/mg protein/minute and represent an average of two separate measurements performed in duplicate.

Immunoprecipitation and Immunoblotting. Cells were lysed in 1 ml of RIPA buffer (10 mM Tris-HCl, Ph 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1% sodium deoxycholate, 0.1% SDS, 1.2% aprotinin, 5 μM leupeptin, 4 μM antipain, 1mM phenylmethylsulfonyl fluoride, 0.1 mM Na_3VO_4), and 300-500 μg were incubated 1 h to overnight with 1-4 μg primary Ab, for 1 h with 40 μl protein G-sepharose beads (Amersham Pharmacia Biotech., Piscataway, NJ) at 4°C, and immunoprecipitates were washed with RIPA buffer, resuspended in 2x Laemmli sample buffer, resolved on 8% or 10% SDS-PAGE and transferred to nitrocellulose. Western blot analyses were performed as described (15) using 1:2000 dilution of Ab. For Far Western analysis, membranes were incubated with 1 μg/ml GST fusion protein containing the C-terminal Src-homology (SH) 2 domain of p85 subunit of PI3-K (Upstate Biotechnology) for 2 h and then Western blotted using the anti-GST mAb (Santa-Cruz Biotechnology, 1:2000) and anti-mouse secondary antibody. Relative intensities of each band were measured by NIH image and levels of phosphorylated protein relative to total protein calculated as the phosphorylation ratio. All experiments were performed on at least 2 separate occasions using different cell lysates with similar results.

Cell Migration Assays. Migration experiments were performed using 10 mm Tissue Culture

Inserts with 8.0 μm polycorborate membranes (NUNC Brand Products, Naperville, IL) as described (2) with minor modifications. Membranes were coated with 100 μl of 1 mg/ml of MATRIGEL matrix (Becton Dickinson Labware, Bedford, MA) for 1 hr at room temperature, followed by 2% BSA in PBS as a blocking reagent for 1 h. Following overnight culture in serum-free media (LNCaP, TSU-Pr1, DU145 and PC-3) or culture for 48 h in media containing tetracycline and/or CGS24592 (WT-5, TN-12 and M-22), cells were harvested, washed and resuspended in serum-free RPMI1640 and added to the upper chambers at a concentrations of $5x10^4$ cells per well. Media in upper and lower chambers contained serum-free RPMI1640 with bombesin or ET-1 at appropriate concentrations or 10% FCS (LNCaP, TSU-Pr1, DU145 and PC-3); or serum-free RPMI1640 with or without tetracycline (WT-5, TN-12 and M-22). CGS24592, rNEP and PP2 were added to cells in upper chambers and to the media in lower chambers 2 h prior to other reagents. Cells were allowed to migrate for 10 h at 37°C in a humidified atmosphere containing 5% CO_2. Membranes were fixed and stained using a Diff-Quik Stain Set (Dade Diagnostic of P.R. Inc, Aguada, PR). Non-migrated cells on the upper side of the membrane were removed with a cotton swab. Migrated cells attached to the lower side of the membrane were enumerated using a light microscope at 10X magnification. Each data point represents the average cell number of 6 independent microscopic fields from a single experiment. Statistical analyses were performed using an unpaired t-test. P values less than 0.005 are reported as <0.005. All migration assays were performed on three separate occasions with similar results.

Results

NEP enzymatic activity inhibits FAK phosphorylation and cell migration in PC cells. Focal

adhesion kinase (FAK) is an important component of integrin-mediated signal transduction and

plays a key role in modulating cell migration of PC cells (2, 16). FAK is activated through

tyrosine phosphorylation, which occurs following cell adhesion to ECM proteins and ligand

binding of growth factors to their cell-surface receptors, and FAK phosphorylation on tyrosine-

397 is necessary for FAK-promoted cell migration (17). Anti-pTyr Western blotting of FAK

immunoprecipitates revealed low levels of FAK phosphorylation and a slightly lower amount of

total FAK protein in androgen-sensitive LNCaP cells compared to the levels of FAK

phosphorylation and FAK protein in androgen-independent, TSU-Pr1, DU-145 and PC-3 PC

cells (Figure 1A, panels 2 and 3), which inversely correlated with the amount of NEP protein in

these cells (Figure 1A, panel 1). TSU-Pr1, DU-145 and PC-3 cells ability to migrate through

Matrigel was 9.7, 9.1, and 12.2-fold higher, respectively, then LNCaP cells (p<0.005 for all three

cell lines).

NEP neuropeptide substrates bombesin and ET-1 stimulate phosphorylation of FAK (18,

19) and promote the migration of highly metastatic PC cells through ECM but have minimal

effects on LNCaP cells (20). Inhibition of NEP enzymatic activity in LNCaP cells cultured in

RPMI containing FCS using the NEP competitive enzyme inhibitor CGS24592 for 2 h at

concentrations ranging from 0 to 100 nM resulted in increased FAK phosphorylation on tyrosine

in a dose dependent fashion (Figure 1B). Pretreatment of LNCaP cells cultured in RPMI without

FCS with 10 nM CGS24592 for 2 h before the addition of 10 nM ET-1 for 20 min resulted in a

30-fold increase in FAK phosphorylation (Figure 1C, lane 4), compared to ET-1 or CGS24592

8

alone (lanes 2 and 3). CGS24592 (10 nM) incubation in RPMI containing FCS resulted in 4.3-fold increase in LNCaP migrated cell number compared with untreated control cells (p<0.005).

Similar experiments investigating the effect of rNEP on FAK phosphorylation and cell migration in TSU-Pr1 cells cultured in media containing FCS showed rNEP can inhibit FAK phosphorylation and cell migration in a time and dose dependent fashion (data not shown). Bombesin or ET-1 stimulate a 22 and 26-fold increase, respectively, in the levels of phosphorylated FAK in TSU-Pr1 cells cultured in media without serum for 24 h (Figure 1D, lanes 2 and 5), which is significantly inhibited by pretreatment with rNEP for 2 h (lanes 3 and 6). Furthermore, bombesin and ET-1 induced cell migration of TSU-Pr1 cells (3.6-fold for bombesin, p<0.005; 4.4-fold for ET-1, p<0.005) is blocked by rNEP (Figure 1E). Taken together, these data on LNCaP and TSU-Pr1 cells suggest that loss of NEP expression in PC cells results in increased FAK phosphorylation and cell migration, which is mediated by neuropeptide NEP substrates.

Overexpression of cell-surface NEP inhibits FAK phosphorylation and cell migration. To further characterize the role of cell-surface NEP in regulating FAK phosphorylation and cell migration, we used the inducible tetracycline-regulatory gene expression system to establish cell lines in parental TSU-Pr1 cells which express wild-type NEP (WT-5), catalytically inactive NEP (M-22) and a control cell line containing the pTRE empty vector (TN-12) (6). Western analysis showed that NEP protein was expressed in both WT-5 and M-22 cells but not TN-12 cells 48 h after tetracycline withdrawal (Figure 2A). Enzyme assays showed high levels of NEP specific activity in total cell lysates from WT-5 cells (505.3 ± 82.7 pmol/μg/min) compared with minimal activity in lysates from TN-12 (2.0 ± 0.7 pmol/μg/min) or M-22 (7.3 ± 4.2 pmol/μg/min) cells following

9

tetracycline withdrawal. NEP expression in WT-5 cells resulted in a ~95% decrease in FAK

phosphorylation (Figure 2B, lane 3 compared to lane 1), but did not alter FAK phosphorylation

in TN-12 cells (lane 6 compared to lane 5). However, in M-22 cells, induced expression of

catalytically inactive NEP also resulted in a partial inhibition of FAK phosphorylation (lane 8

compared to lane 7). Likewise, incubation of WT-5 cells following tetracycline withdrawal with

100 nM CGS24592 only partially inhibited FAK phosphorylation (lane 4) despite that NEP

enzymatic activity is completely inhibited by CGS24592 at this concentration (NEP enzyme

specific activity 6.5 ± 3.3 pmol/μg/min). CGS24592 did not alter FAK phosphorylation when

NEP expression was suppressed by tetracycline (lane 2). As illustrated in Figure 2C, cell

migration studies performed using the identical conditions depicted in Figure 2B demonstrated

that expression of NEP in WT-5 cells resulted in a >90% decrease compared with control

($p < 0.005$), which was partially reversed by 100 nM CGS24592. NEP expression in M-22 cells

resulted in a 54% decrease ($p < 0.005$), which was less than that observed in WT-5 cells but

comparable to the inhibition observed in WT-5 cells cultured with CGS24592. These data show

that blocking NEP enzyme activity through either a competitive enzyme inhibitor or gene

mutation does not completely abrogate the ability of cell-surface NEP to inhibit FAK

phosphorylation and cell migration and suggest that expression of the NEP protein at the cell

surface can induce dephosphorylation of FAK through a mechanism distinct from NEP's

catalytic function.

NEP enzyme activity inhibits the formation of cSrc and FAK protein complexes. The ability of

FAK to transmit signals to downstream targets is dependent on its interaction with several

intracellular signaling molecules including Src family kinases (21, 22) and PI3-K (23). Tyr-397

10

has been identified as the major site of FAK autophosphorylation and the binding site for the SH2 domains of cSrc (24) and the p85 subunit of PI3-K (23). Substitution of Tyr-397 with Phe abolishes FAK-promoted cell migration (17), implicating a role for cSrc and PI3-K in cell migration. The association of FAK with cSrc is also critical in promoting bombesin-mediated FAK phosphorylation and cell migration via G-protein coupled receptor (GPCR) pathways (19, 25). Western blotting of cSrc immunoprecipitates from PC cells revealed that FAK coimmunoprecipitates with cSrc in three androgen-independent PC cell lines but not in LNCaP cells (Figure 3A), despite the fact that LNCaP cells express abundant amounts of cSrc protein (Figure 3A, lower panel) and FAK protein (Figure 1A, panel 3). The FAK-cSrc co-immunoprecipitation was partially restored by culturing LNCaP cells in media containing FCS with CGS24592 (data not shown), or in serum-free media with CGS24592 followed by ET-1 (Figure 3B, lane 4). In TSU-Pr1 cells, the specific Src kinase inhibitor PP2 inhibited bombesin-induced FAK phosphorylation (Figure 3C, lane 3 compared to lane 2). 10 μM PP2 also inhibited FAK phosphorylation in TSU-Pr1 cells cultured in media containing FCS (lane 5 compared with lane 4). This decrease in FAK phosphorylation was not affected by the addition of 50 μg/ml of rNEP for 2 h prior to the addition of PP2 (lane 6). Cell migration assays showed that treatment of TSU-Pr1 cells with 10 μM PP2 resulted in 45% decrease in migrated cell number compared with untreated control (p=0.0025), and that the addition of 50 μg/ml rNEP did not significant affect the migrated cell number compared with PP2 treatment (p=0.3907). Finally, assessment of the association of cSrc and FAK in WT-5, M-22 and TN12 cells before and after tetracycline withdrawal showed that FAK and cSrc co-immunoprecipitated in WT-5 (Figure 3D, lane 1), M-

11

22 (lane 7) and TN-12 cells (lane 5) when cultured in tetracycline which suppresses NEP expression. Expression of NEP by tetracycline withdrawal completely inhibited the association of FAK and cSrc in WT-5 cells (lane 3), but not in M22 cells (lanes 8). The inhibition of FAK-cSrc co-immunoprecipitation in WT-5 cells (lane 3) was reversed by culturing in the presence of CGS24592 (lane 4). Taken together, these data show that 1) NEP inhibition of neuropeptide-mediated FAK phosphorylation and cell migration results in part from lack of association of FAK with c-Src; 2) neuropeptide induction of FAK phosphorylation requires cSrc-kinase activity; 3) catalytic inactivation of NEP substrates does not further promote FAK dephosphorylation or inhibition of cell migration if cSrc-kinase activity is inhibited; and 4) the association of FAK and cSrc is inhibited by expression of enzymatically active NEP protein, but not by expression of catalytically inactive NEP protein.

NEP complexes with the p85 subunit of PI3-K. Expression of catalytically inactive NEP protein in M22 cells partially inhibited FAK phosphorylation and cell migration, but did not alter the association of FAK and cSrc suggesting an alternative pathway of NEP regulation of FAK phosphorylation and cell migration. Previous reports indicate that PI3-K interacts with and phosphorylates FAK in FAK-promoted cell migration (26). Western analysis of immunoprecipitates of the p85 subunit of PI3-K showed that p85 coimmunoprecipitated with FAK in androgen-independent PC cells but not in LNCaP cells (Figure 4A). In contrast to the association of FAK with cSrc, the FAK-p85 co-immunoprecipitation could not be restored by incubating LNCaP cells with CGS24592 or CGS24592 and ET-1, indicating that this protein-protein interaction was not dependent on NEP enzyme activity or NEP substrates (data not shown). Expression of wild type NEP (Figure 4B, lane 3) or mutated NEP (lane 8) blocked the

12

association of p85 with FAK, which could not be reversed by culturing in the presence of the NEP enzyme inhibitor CGS24592 (lane 4). These experiments showed NEP expression inhibits the association of p85 with FAK. Western blotting using an NEP Ab of immunoprecipitates of the p85 subunit of PI3-K revealed NEP co-immunoprecipitated with p85 in LNCaP cells but not other PC cell lines (Figure 5A, panel 1), which was confirmed by Western blotting of NEP immunoprecipitates using an p85 Ab (panel 4). Identical results were obtained in WT-5 and M-22 cells in which NEP and p85 co-immunoprecipitates could be detected when either wild-type or mutated NEP was expressed following tetracycline withdrawal (Figure 5B, panels 1 and 4). Thus, these data suggest that NEP protein complexes with the p85 subunit of PI3-K and inhibits the association of p85 with FAK, and that expression of catalytically inactive NEP protein in M22 cells partially inhibits FAK phosphorylation and cell migration by NEP complexing with p85.

Lyn kinase mediates p85-NEP interaction. NEP's cytoplasmic domain lacks any characteristic structural motifs such as a SH2 domain or a proline-rich region required for associating with p85, suggesting that NEP and p85 do not directly associate. NEP is reported to associate with the Src family kinase member Lyn and an unknown 75-85 kD protein in B-cells (9, 10). Our study showed that Lyn was also detected by Western blotting of NEP immunoprecipitates with a Lyn Ab in NEP-expressing LNCaP, WT-5 and M-22 cells (data not shown). Similarly, Western blotting of Lyn immunoprecipitates with an NEP Ab detected NEP (Figure 6A and 6B, panel 1). Moreover, p85 co-immunoprecipitated with NEP and Lyn (panel 2), and anti-pTyr Western blotting of Lyn immunoprecipitates showed that Lyn phosphorylation is increased in cells expressing NEP (panel 3 compared to total Lyn protein in panel 4). Lyn directly associates with

p85 when NEP is expressed as shown by binding of GST-p85 SH2 to Lyn immunoprecipitated from M-22 cells expressing NEP following tetracycline withdrawal (Figure 6C). Finally, an analysis of immunoprecipitates of p85 from M-22 cells at 8, 16 and 24 h following tetracycline withdrawal indicated that the p85-FAK association (Figure 6D, panel 1) decreased as the association of p85 with both NEP (panel 2) and Lyn (panel 3) increased. Taken together, these results suggest that NEP associates with and induces Lyn phosphorylation, which in turn directly bind to p85 of PI3-K, resulting in complex of NEP-Lyn-PI3-K, which competitively blocks FAK-PI3-K interaction, leading to a decrease in FAK phosphorylation and cell migration.

Discussion

The involvement of NEP loss in the transition from androgen-dependent PC to androgen-independent PC has only recently been recognized (6). We previously reported that NEP expression is decreased in many androgen-independent PCs in vivo (6); that the NEP gene is transcriptionally activated by androgen and that androgen-withdrawal results in decreased NEP expression (6, 15); and that hypermethylation of the 5' CpG NEP island in the NEP promoter is associated with loss of NEP expression (27). These results suggest a model in which decreased NEP expression is facilitated by the elimination of androgens or hypermethylation of the NEP promoter, contributing to the development of neuropeptide-mediated, androgen-independent PC cell growth. The current study aimed at elucidating the mechanisms of NEP action on PC cells reveals a dual function of the NEP protein (Figure 7). NEP inhibits neuropeptide-mediated stimulation of the association of cSrc and FAK through catalytic inhibition of its neuropeptide substrates such as bombesin and ET-1, and NEP inhibits the association of PI3-K with FAK by indirectly associating with PI3-K through Lyn kinase. These effects on FAK phosphorylation directly correlate with PC cell migration, and suggest that NEP normally functions to regulate PC cell migration and possibly other FAK-associated processes.

The effects of NEP on bombesin and ET-1 are not surprising as these neuropeptides induce FAK activation via a cSrc-dependent pathway (19, 28). The association of FAK with cSrc plays a major role in FAK-mediated signal transduction (29), inducing phosphorylation of FAK at multiple sites, including Tyr 577 and Tyr 925, resulting in maximal FAK activity (29, 30). We show that cell migration, FAK phosphorylation and the association of cSrc with FAK 1) increases in NEP-negative, androgen-independent PC cells treated with neuropeptide; 2) increases

in NEP-expressing LNCaP cells cultured with a specific NEP enzyme inhibitor plus neuropeptide; and 3) decreases with the addition of rNEP or the expression of cell-surface NEP in androgen-independent PC cells. These results suggest that NEP enzymatic activity inhibits FAK phosphorylation and cell migration by affecting neuropeptide-induced interaction of FAK with cSrc.

Expression of a mutated, catalytically inactive cell-surface NEP protein also inhibited FAK phosphorylation and cell migration although to a lesser degree than observed following expression of wild-type NEP. This observation suggested another mechanism of NEP action distinct from its enzymatic function, and led us to examine whether PI3-K, which has been implicated in promoting cell migration, was involved. The concept that NEP protein independent of its catalytic activity is involved in signal transduction is supported by two recent studies. NEP/CD10 expression is a marker for apoptosis in T-cells and the apoptotic capacity is not dependent of NEP catalytic activity (11), and NEP/CD10 expression negatively affects integrin-mediated signal transduction in NEP/CD10 positive B lymphocyte leukemia cells (31). Our observation that NEP protein associates with the p85 subunit of PI3-K was unexpected. This association occurs in LNCaP cells and in androgen-independent PC cells in which NEP expression is induced. Expression of NEP in these cells is also associated with a loss of the interaction of p85 with FAK. Numerous studies have suggested that FAK associates with PI3-K (23, 26, 32) and this association affects the integrity of the actin cytoskeleton (32) and cell migration (26), although it remains controversial whether PI3-K-mediated cell migration is dependent on FAK (26, 33). Thus, we hypothesize that in PC cells NEP's effect on cell migration and FAK phosphorylation is also mediated by inhibition of FAK-PI3-K interaction.

16

Although we have shown that tyrosine-phosphorylated Lyn which is induced following NEP expression inhibits FAK-PI3-K interactions by associating directly with p85, we did not define the enzyme which phosphorylates Lyn, or how an NEP-phosphorylated Lyn complex inhibits PI3-K associating with FAK. NEP is present in detergent-resistant glycosylphosphatidylinosotol (GPI)-microdomains (10) which are enriched in various signaling molecules, including p85 and its substrate PIP2 (34). NEP in GPI-microdomains co-immunoprecipitates with Lyn and an unknown 75-85 kD protein (9, 10), which we speculate is p85. PI3-K located in focal contacts can reactivate integrin function through inside-out signaling (35, 36), suggesting that PI3-K associates with FAK in focal contacts and also regulates FAK-mediated cell migration pathways. In addition, Lyn has previously been implicated as a negative regulator of PI3-K signaling (37, 38). Thus, we speculate that NEP expression results in phosphorylated Lyn binding PI3-K causing PI3-K to translocate from focal contacts (which contain integrin-FAK complexes) thereby inhibiting FAK-PI3-K interactions. Preliminary studies support this hypothesis (unpublished data).

In summary, we describe a novel dual function of NEP to inhibit FAK phosphorylation and cell migration by blocking FAK association with cSrc and PI3-K, suggesting a complex system in which NEP plays an important role. Recent studies showing that bombesin signaling crosstalks with the EGF receptor (39), and that ET-1 acts as a survival factor (40) suggest that NEP may be involved in other critical pathways. Further studies will help define the role of NEP and its substrates in regulating PC development and progression.

Figure 1. NEP expression, FAK phosphorylation and cell migration in PC cells. A. (Panel 1):

Total cell lysates (20 μg) from PC cells were analyzed for NEP protein by Western blot as

described in Materials and Methods using the anti-NEP Ab 5B5. (Panel 2): 300 μg of PC total

cell lysates were immunoprecipitated with anti-FAK Ab C-20, separated by SDS-PAGE,

transferred to nitrocellulose and Western blotted with anti-pTyr mAb PY20. (Panel 3): The same

blot shown in panel 2 was stripped and reprobed with Ab C-20 for FAK protein. B. LNCaP

cells cultured in RPMI containing FCS and the NEP competitive enzyme inhibitor CGS24592

(CGS) for 2 h at concentrations ranging from 0 to 100 nM were immunoprecipitated with anti-

FAK Ab C20, separated by SDS-PAGE, transferred to nitrocellulose and Western blotted

consecutively with mAb PY20 (upper panel) and Ab C-20 (lower panel). C. LNCaP cells

cultured in RPMI without serum (lane 1), 10 nM ET-1 for 20 min (lane 2), 10 nM CGS24592 for

2 h (lane 3), or 10 nM CGS24592 for 2 h followed by 10 nM ET-1 for 20 min (lane 4). Cells

were lysed and analyzed as described in 1B. D. TSU-Pr1 cells were cultured in media without

FCS for 24 hr (Lanes 1 and 4), followed by the addition of 10 nM bombesin (Bomb., lane 2) or

10 nM ET-1 (lane 5) for 20 min; or by the addition of 50 μg/ml of rNEP for 2 h, and then

bombesin (lane 3) or 10 ET-1 (lane 6) for 20 min. Cells were lysed and analyzed as described

above. E. Cell migration assays were performed in conditions identical to 1D. Bars represent SD.

Experiments were repeated three times with similar results.

Figure 2. Overexpression of cell-surface NEP inhibits FAK phosphorylation and cell migration.

A. TSU-Pr1-derived cell lines containing wild type NEP (WT-5), control empty vector (TN-12)

or mutated, enzymatically inactive NEP (M-22) were cultured with (+) and without (-) 1 μg

tetracycline (Tet). Total cell lysates (20 μg) were analyzed for NEP protein by Western blot as described in Materials & Methods using the anti-NEP Ab 5B5. B. WT-5, TN-12 and M-22 cells were cultured with (+) and without (-) 1 μg tetracycline (with the addition of 100 nM CGS24592 in lanes 2 and 4). Cells were lysed and analyzed as described in Figure 1B legend. C. Cell migration assays were performed using the identical conditions described in 2B. Bars represent SD. Experiments were repeated twice with similar results.

Figure 3. NEP enzyme activity inhibits the formation of cSrc and FAK protein complexes. A. Upper panel: 500 μg of PC total cell lysates were immunoprecipitated with anti-cSrc Ab SRC-2, separated by SDS-PAGE, transferred to nitrocellulose and Western blotted with anti-FAK Ab C20. A-Lower panel: The same blot was stripped and reprobed with mAb B12 for cSrc protein. B. Cell lysates derived from LNCaP cells cultured in media without FCS (lane 1), 10 nM ET-1 for 20 min (lane 2), 10 nM CGS24592 for 2 h (lane 3) or CGS24592 followed by ET-1 (lane 4) were analyzed as described above. C. (left) TSU-Pr1 cells were cultured in serum-free media (lanes 1) with the addition of 10 nM bombesin for 20 min (lane 2), or 10 μM of Src kinase inhibitor PP2 for 30 min prior to the addition bombesin (lane 3). (right) TSU-Pr1 cells were cultured in media containing 10% FCS (lane 4), 10 μM of PP2 (lane 5), or 50 μg/ml of rNEP for 2 h prior to PP2. Cells were lysed and analyzed as described in Figure 1B legend. D. WT-5, TN-12 and M-22 cells were cultured with (+) and without (-) 1 μg tetracycline for 48 h (with the addition of 100 nM CGS24592 in lanes 2 and 4). Cells were lysed and analyzed as described in above.

Figure 4. NEP inhibits the formation of p85-FAK protein complexes. A. 500 μg of total cell

lysates were immunoprecipitated with anti-p85 Ab, separated by SDS-PAGE, transferred to nitrocellulose and Western blotted with anti-FAK Ab C-20 or anti-p85 Ab. B. WT-5, TN-12 and M-22 cells were cultured with (+) and without (-) 1 µg tetracycline (with the addition of 100 nM CGS24592 in lanes 2 and 4). Cells were lysed and analyzed as described above.

Figure 5. NEP complexes with the p85 subunit of PI3-K. A. 500 µg of total cell lysates were immunoprecipitated with anti-p85 Ab, anti-NEP Ab J5 or control Ab (Cont.), separated by SDS-PAGE, transferred to nitrocellulose and Western blotted with anti-NEP Ab 5B5 or anti-p85 Ab. B. Cell lysates derived from WT-5, TN-12 and M-22 cells cultured with (+) and without (-) 1 µg tetracycline were immunoprecipitated with anti-p85 Ab, anti-NEP Ab J5 or control Ab and analyzed as described above. Total cell lysate (TCL) from LNCaP cells was positive control.

Figure 6. Phosphorylated Lyn associates with NEP and p85. A. 500 µg of total cell lysates were immunoprecipitated with rabbit anti-Lyn Ab or control rabbit Ab, separated by SDS-PAGE, transferred to nitrocellulose and Western blotted with anti-NEP Ab, anti-p85 Ab, anti-pTyr Ab or mouse anti-Lyn Ab. B. WT-5, TN-12 and M-22 cells were cultured with (+) and without (-) 1 µg tetracycline. Cells were lysed and analyzed as described above. C. Upper panel: M-22 cells were cultured with or without (-) 1 µg tetracycline for 48 h. Cells were lysed and Far Western blotted as described in Materials and Methods. FAK immunoprecipitate of M-22 cells was used as a positive control for C-terminal SH2 domain of p85 to bind. GST; 0.1 µg recombinant GST protein used as a positive control for anti-GST Ab. Cont.; negative control by rabbit control Ab. IgGL; IgG light chain. C. Lower panel: The same blot shown in upper panel was stripped and reprobed with mouse anti-Lyn Ab. D. M-22 cells were cultured without (-) 1

μg tetracycline for various time periods. Cell lysates were immunoprecipitated with rabbit anti-p85 Ab, separated by SDS-PAGE, transferred to nitrocellulose and Western blotted with anti-FAK Ab, anti-NEP Ab, mouse anti-Lyn Ab or anti-p85 Ab.

Figure 7. NEP Effect on FAK-mediated cell migration. Neuropeptides such as bombesin and ET-1 promote the association of cSrc with FAK, resulting in phosphorylation of FAK at multiple sites including Tyr 577 and Tyr 925. NEP catalytically inactivates these neuropeptides and inhibits neuropeptides-induced, FAK-mediated cell migration. PI3-K also associate with FAK in focal contacts which may further activate FAK–mediated cell migration. NEP complexes with phosphorylated Lyn and binds the p85 subunit of PI3-K, competitively inhibiting the association of p85 with FAK, resulting in decreased FAK-mediated cell migration. GPI-AP; GPI-anchored protein.

REFERENCES

1.   Sehgal, I., S. Powers, B. Huntley, G. Powis, M. Pittelkow, and N.J. Maihle. 1994. Neurotensin is an autocrine trophic factor stimulated by androgen withdrawal in human prostate cancer. *Proc Natl Acad Sci U S A.* 91:4673-4677.

2.   Aprikian, A.G., L. Tremblay, K. Han, and S. Chevalier. 1997. Bombesin stimulates the motility of human prostate-carcinoma cells through tyrosine phosphorylation of focal adhesion kinase and of integrin-associated proteins. *Int J Cancer.* 72:498-504.

3.   Nelson, J.B., and M.A. Carducci. 2000. Small bioactive peptides and cell surface peptidases in androgen- independent prostate cancer. *Cancer Invest.* 18:87-96.

4.   Shipp, M.A., G.E. Tarr, C.Y. Chen, S.N. Switzer, L.B. Hersh, H. Stein, M.E. Sunday, and E.L. Reinherz. 1991. CD10/neutral endopeptidase 24.11 hydrolyzes bombesin-like peptides and regulates the growth of small cell carcinomas of the lung. *Proc Natl Acad Sci U S A.* 88:10662-10666.

5.   Shipp, M.A., and A.T. Look. 1993. Hematopoietic differentiation antigens that are membrane-associated enzymes: cutting is the key! *Blood.* 82:1052-1070.

6.   Papandreou, C.N., B. Usmani, Y. Geng, T. Bogenrieder, R. Freeman, S. Wilk, C.L. Finstad, V.E. Reuter, C.T. Powell, D. Scheinberg, C. Magill, H.I. Scher, A.P. Albino, and D.M. Nanus. 1998. Neutral endopeptidase 24.11 loss in metastatic human prostate cancer contributes to androgen-independent progression. *Nat Med.* 4:50-57.

7.   Harrison, N.K., K.E. Dawes, O.J. Kwon, P.J. Barnes, G.J. Laurent, and K.F. Chung. 1995. Effects of neuropeptides on human lung fibroblast proliferation and chemotaxis. *Am J Physiol.* 268:L278-283.

8.   Hofman, P., E. Selva, G. Le Negrate, L. d'Andrea, S. Guerin, B. Rossi, and P. Auberger. 1998. CD10 inhibitors increase f-Met-Leu-Phe-induced neutrophil transmigration. *J Leukoc Biol*. 63:312-320.

9.   Ganju, R.K., R.G. Shpektor, D.G. Brenner, and M.A. Shipp. 1996. CD10/neutral endopeptidase 24.11 is phosphorylated by casein kinase II and coassociates with other phosphoproteins including the lyn src- related kinase. *Blood*. 88:4159-4165.

10.   Angelisova, P., K. Drbal, V. Horejsi, and J. Cerny. 1999. Association of CD10/neutral endopeptidase 24.11 with membrane microdomains rich in glycosylphosphatidylinositol-anchored proteins and Lyn kinase [letter]. *Blood*. 93:1437-1439.

11.   Cutrona, G., N. Leanza, M. Ulivi, G. Melioli, V.L. Burgio, G. Mazzarello, G. Gabutti, S. Roncella, and M. Ferrarini. 1999. Expression of CD10 by human T cells that undergo apoptosis both in vitro and in vivo. *Blood*. 94:3067-3076.

12.   De Lombaert, S., M.D. Erion, J. Tan, L. Blanchard, L. el-Chehabi, R.D. Ghai, Y. Sakane, C. Berry, and A.J. Trapani. 1994. N-Phosphonomethyl dipeptides and their phosphonate prodrugs, a new generation of neutral endopeptidase (NEP, EC 3.4.24.11) inhibitors. *J Med Chem*. 37:498-511.

13.   Yang, X.F., C. Chatellard, C. Lazure, P. Crine, and G. Boileau. 1994. Insertion of hydrophilic amino acid residues in the signal peptide/membrane anchor domain of neprilysin (neutral endopeptidase- 24.11) results in its cleavage: role of the position of insertion. *Arch Biochem Biophys*. 315:382-386.

14.   Devault, A., C. Nault, M. Zollinger, M.C. Fournie-Zaluski, B.P. Roques, P. Crine, and G. Boileau. 1988. Expression of neutral endopeptidase (enkephalinase) in heterologous COS-

1 cells. Characterization of the recombinant enzyme and evidence for a glutamic acid residue at the active site. *J Biol Chem.* 263:4033-4040.

15.   Shen, R., M. Sumitomo, J. Dai, A. Harris, D. Kaminetzky, M. Gao, K.L. Burnstein, and D.M. Nanus. 2000. Androgen-induced growth inhibition of androgen receptor expressing androgen-independent prostate cancer cells is mediated by increased levels of neutral endopeptidase. *Endocrinology.* 141:1699-1704.

16.   Zheng, D.Q., A.S. Woodard, M. Fornaro, G. Tallini, and L.R. Languino. 1999. Prostatic carcinoma cell migration via alpha(v)beta3 integrin is modulated by a focal adhesion kinase pathway. *Cancer Res.* 59:1655-1664.

17.   Cary, L.A., J.F. Chang, and J.L. Guan. 1996. Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. *J Cell Sci.* 109:1787-1794.

18.   Zachary, I., and E. Rozengurt. 1992. Focal adhesion kinase (p125FAK): a point of convergence in the action of neuropeptides, integrins, and oncogenes. *Cell.* 71:891-894.

19.   Salazar, E.P., and E. Rozengurt. 1999. Bombesin and platelet-derived growth factor induce association of endogenous focal adhesion kinase with Src in intact Swiss 3T3 cells. *J Biol Chem.* 274:28371-28378.

20.   Tremblay, L., W. Hauck, A.G. Aprikian, L.R. Begin, A. Chapdelaine, and S. Chevalier. 1996. Focal adhesion kinase (pp125FAK) expression, activation and association with paxillin and p50CSK in human metastatic prostate carcinoma. *Int J Cancer.* 68:164-171.

21.   Schlaepfer, D.D., S.K. Hanks, T. Hunter, and P. van der Geer. 1994. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase.

25

Nature. 372:786-791.

22.   Schaller, M.D., J.D. Hildebrand, and J.T. Parsons. 1999. Complex formation with focal adhesion kinase: A mechanism to regulate activity and subcellular localization of Src kinases. *Mol Biol Cell.* 10:3489-3505.

23.   Chen, H.C., P.A. Appeddu, H. Isoda, and J.L. Guan. 1996. Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase. *J Biol Chem.* 271:26329-26334.

24.   Schaller, M.D., J.D. Hildebrand, J.D. Shannon, J.W. Fox, R.R. Vines, and J.T. Parsons. 1994. Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol Cell Biol.* 14:1680-1688.

25.   Rozengurt, E. 1998. Signal transduction pathways in the mitogenic response to G protein- coupled neuropeptide receptor agonists. *J Cell Physiol.* 177:507-517.

26.   Reiske, H.R., S.C. Kao, L.A. Cary, J.L. Guan, J.F. Lai, and H.C. Chen. 1999. Requirement of phosphatidylinositol 3-kinase in focal adhesion kinase- promoted cell migration. *J Biol Chem.* 274:12361-12366.

27.   Usmani, B.A., R. Shen, M. Janeczko, C.N. Papandreou, W.H. Lee, W.G. Nelson, J.B. Nelson, and D.M. Nanus. 2000. Methylation of the neutral endopeptidase gene promoter in human prostate cancers. *Clin Cancer Res.* 6:1664-1670.

28.   Rodriguez-Fernandez, J.L., and E. Rozengurt. 1998. Bombesin, vasopressin, lysophosphatidic acid, and sphingosylphosphorylcholine induce focal adhesion kinase activation in intact Swiss 3T3 cells. *J Biol Chem.* 273:19321-19328.

29.   Schlaepfer, D.D., K.C. Jones, and T. Hunter. 1998. Multiple Grb2-mediated

integrin-stimulated signaling pathways to ERK2/mitogen-activated protein kinase: summation of both c-Src- and focal adhesion kinase-initiated tyrosine phosphorylation events. *Mol Cell Biol.* 18:2571-2585.

30. Calalb, M.B., T.R. Polte, and S.K. Hanks. 1995. Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. *Mol Cell Biol.* 15:954-963.

31. Geijtenbeek, T.B., Y. van Kooyk, S.J. van Vliet, M.H. Renes, R.A. Raymakers, and C.G. Figdor. 1999. High frequency of adhesion defects in B-lineage acute lymphoblastic leukemia. *Blood.* 94:754-764.

32. Guinebault, C., B. Payrastre, C. Racaud-Sultan, H. Mazarguil, M. Breton, G. Mauco, M. Plantavid, and H. Chap. 1995. Integrin-dependent translocation of phosphoinositide 3-kinase to the cytoskeleton of thrombin-activated platelets involves specific interactions of p85 alpha with actin filaments and focal adhesion kinase. *J Cell Biol.* 129:831-842.

33. Danilkovitch, A., A. Skeel, and E.J. Leonard. 1999. Macrophage stimulating protein-induced epithelial cell adhesion is mediated by a PI3-K-dependent, but FAK-independent mechanism. *Exp Cell Res.* 248:575-582.

34. Xavier, R., T. Brennan, Q. Li, C. McCormack, and B. Seed. 1998. Membrane compartmentation is required for efficient T cell activation. *Immunity.* 8:723-732.

35. Nagel, W., L. Zeitlmann, P. Schilcher, C. Geiger, J. Kolanus, and W. Kolanus. 1998. Phosphoinositide 3-OH kinase activates the beta2 integrin adhesion pathway and induces membrane recruitment of cytohesin-1. *J Biol Chem.* 273:14853-14861.

36. Adelsman, M.A., J.B. McCarthy, and Y. Shimizu. 1999. Stimulation of beta1-

integrin function by epidermal growth factor and heregulin-beta has distinct requirements for erbB2 but a similar dependence on phosphoinositide 3-OH kinase. *Mol Biol Cell*. 10:2861-2878.

37.   Satterthwaite, A.B., C.A. Lowell, W.N. Khan, P. Sideras, F.W. Alt, and O.N. Witte. 1998. Independent and opposing roles for Btk and lyn in B and myeloid signaling pathways. *J Exp Med*. 188:833-844.

38.   Craxton, A., A. Jiang, T. Kurosaki, and E.A. Clark. 1999. Syk and Bruton's tyrosine kinase are required for B cell antigen receptor-mediated activation of the kinase Akt. *J Biol Chem*. 274:30644-30650.

39.   Prenzel, N., E. Zwick, H. Daub, M. Leserer, R. Abraham, C. Wallasch, and A. Ullrich. 1999. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature*. 402:884-888.

40.   Eberl, L.P., O. Valdenaire, V. Saintgiorgio, J.F. Jeannin, and L. Juillerat-Jeanneret. 2000. Endothelin receptor blockade potentiates FasL-induced apoptosis in rat colon carcinoma cells. *Int J Cancer*. 86:182-187.
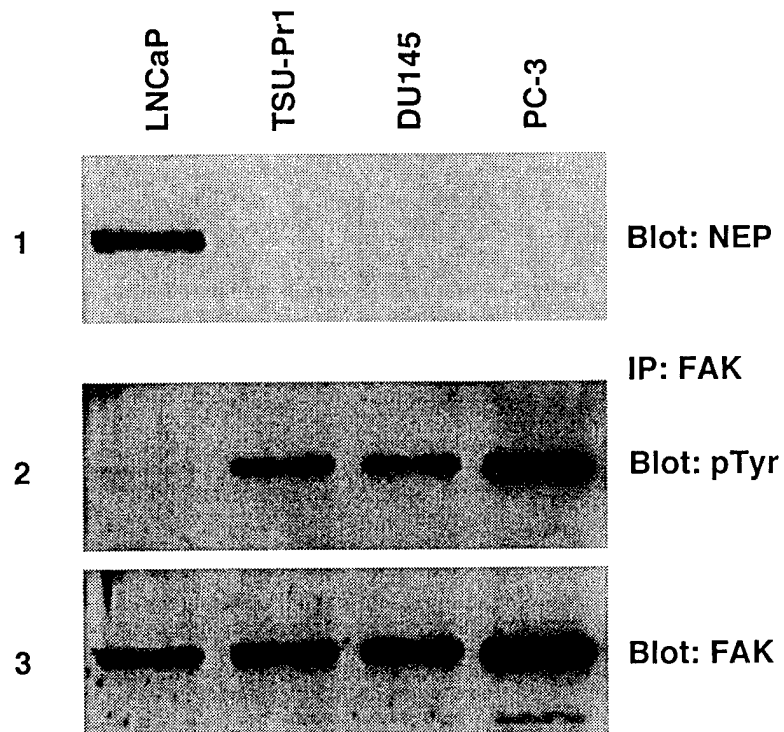
FIGURE 1 A

A
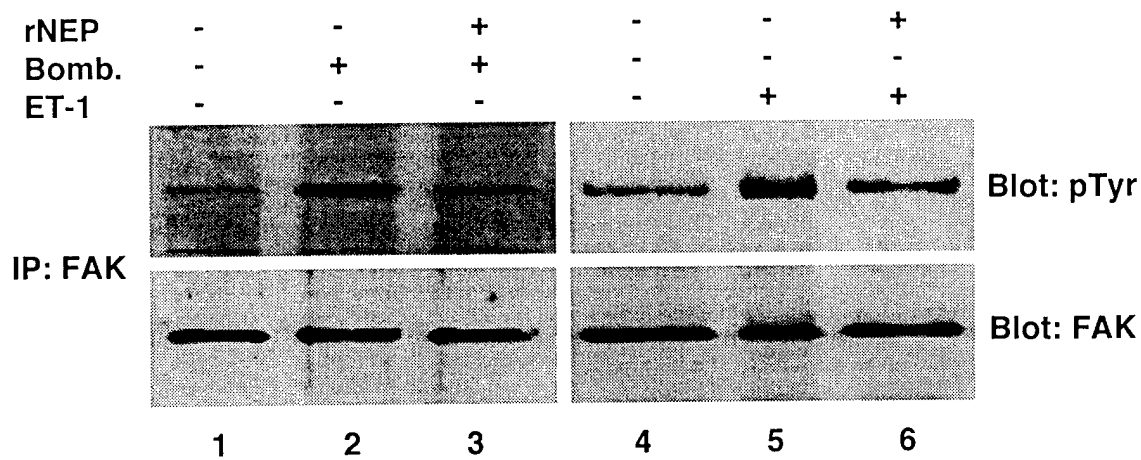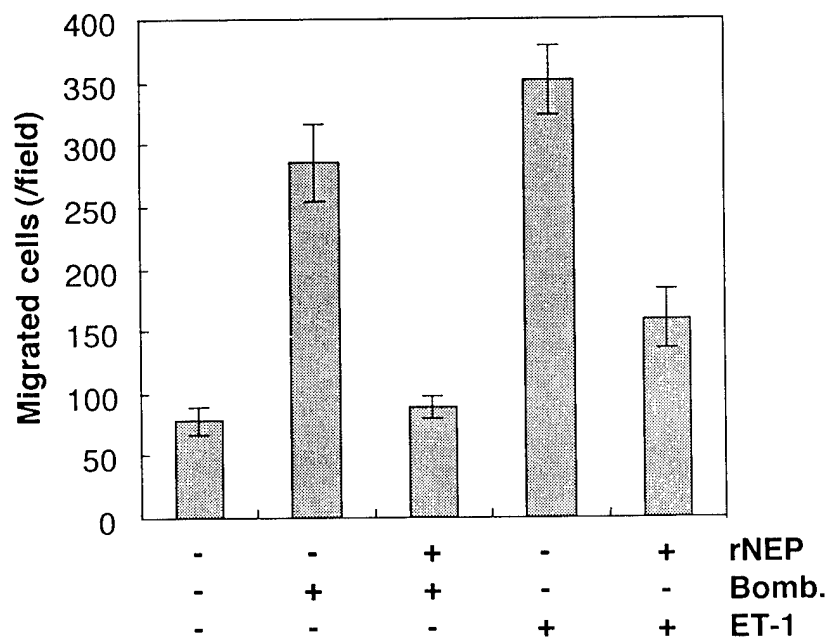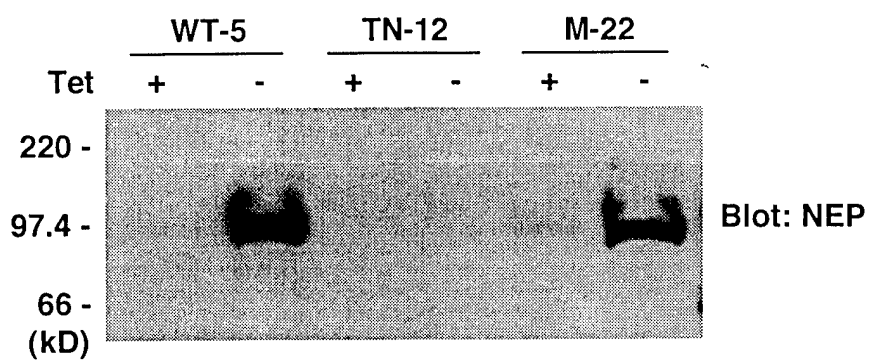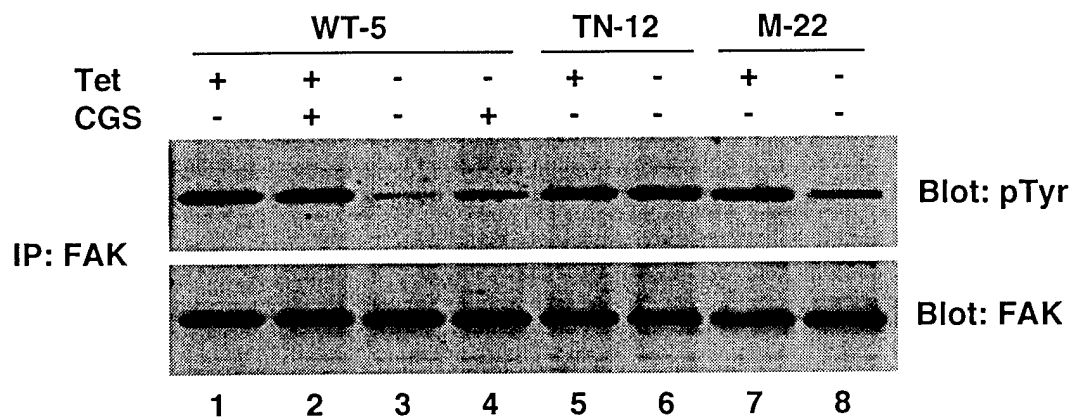
B

10% FCS
_____

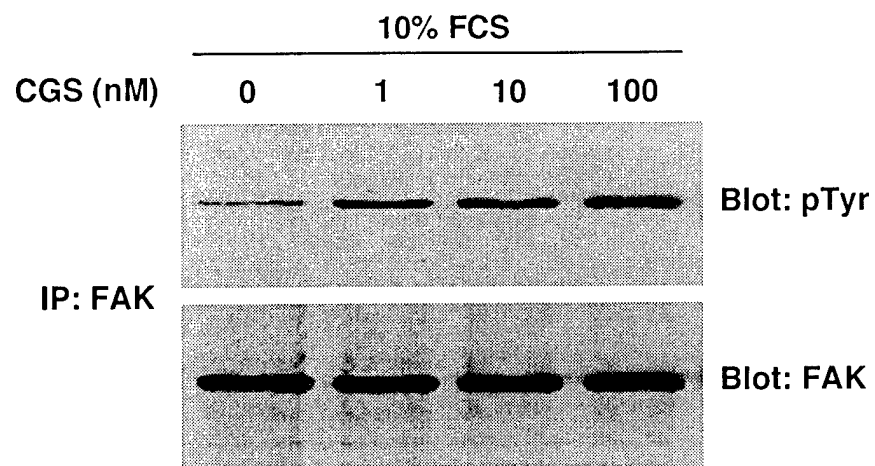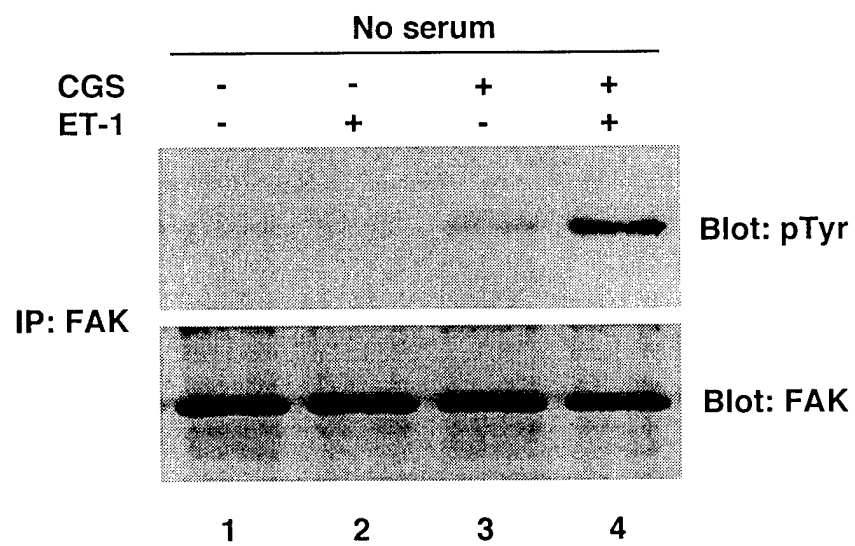CGS (nM)   0   1   10   100

Blot: pTyr

IP: FAK

Blot: FAK

C

No serum
_____

CGS   -   -   +   +
ET-1   -   +   -   +

Blot: pTyr

IP: FAK

Blot: FAK

1   2   3   4

D

E

A

B

C

A

FIGURE 3A

IP: cSrc

LNCaP TSU-Pr1 DU145 PC-3

Blot: FAK

Blot: cSrc

FIGURE 3B

B

LNCaP in no serum

| CGS | - | - | + | + |
| ET-1 | - | + | - | + |

IP: cSrc

Blot: FAK

Blot: cSrc

1   2   3   4

C

TSU-Pr1

	No serum			10% FCS		
Bomb.	-	+	+	-	-	-
PP2	-	-	+	-	+	+
rNEP	-	-	-	-	-	+

IP: FAK

Blot: pTyr

Blot: FAK

1   2   3   4   5   6

D

	WT-5				TN-12		M-22	
Tet	+	+	-	-	+	-	+	-
CGS	-	+	-	+	-	-	-	-

IP: cSrc

Blot: FAK

Blot: cSrc

1   2   3   4   5   6   7   8

A

IP: p85

Blot: FAK

Blot: p85

LNCaP TSU-Pr1 DU145 PC-3

B

	WT-5				TN-12		M-22	
Tet	+	+	-	-	+	-	+	-
CGS	-	+	-	+	-	-	-	-

IP: p85

Blot: FAK

Blot: p85

1   2   3   4   5   6   7   8

FIGURE 5A

B

A

FIGURE 6A

LNCaP TSU-Pr1 DU145 PC-3 LNCaP

1 — Blot: NEP

2 — Blot: p85

3 — Blot: pTyr (Lyn)

4 — Blot: Lyn

IP: Lyn   Cont.

FIGURE 6B

B

	WT-5	TN-12	M-22	
Tet	+ -	+ -	+ -	-

1 — Blot: NEP

2 — Blot: p85

3 — Blot: pTyr (Lyn)

4 — Blot: Lyn

IP: Lyn   Cont.

C

FIGURE 6C

FIGURE 6D

D

FIGURE 7

Activation

Inhibition

Association
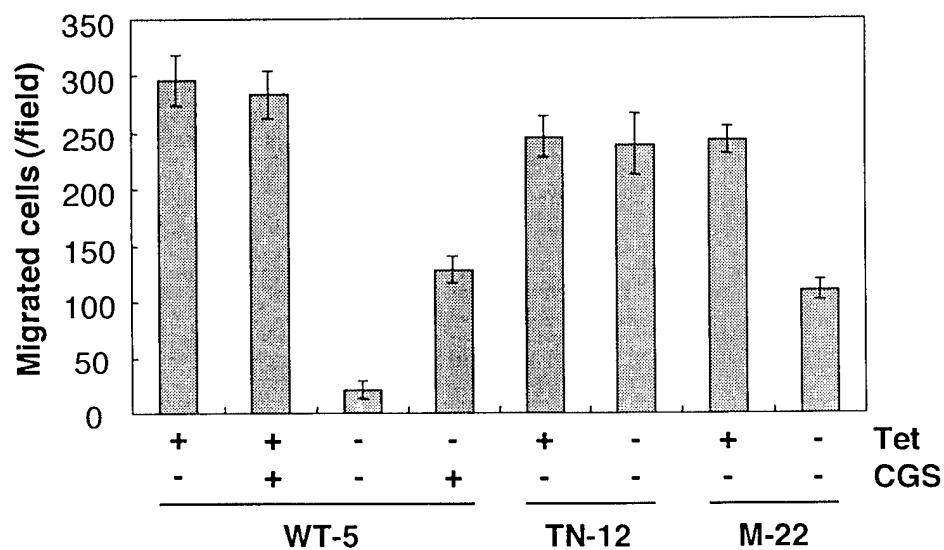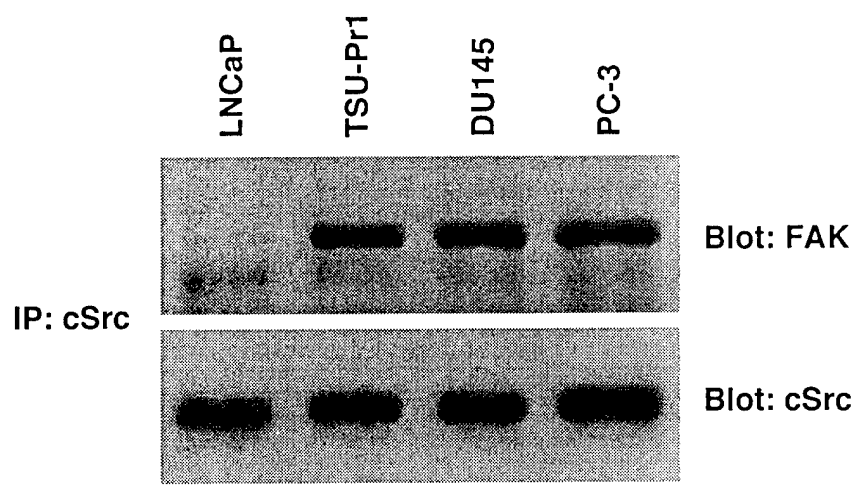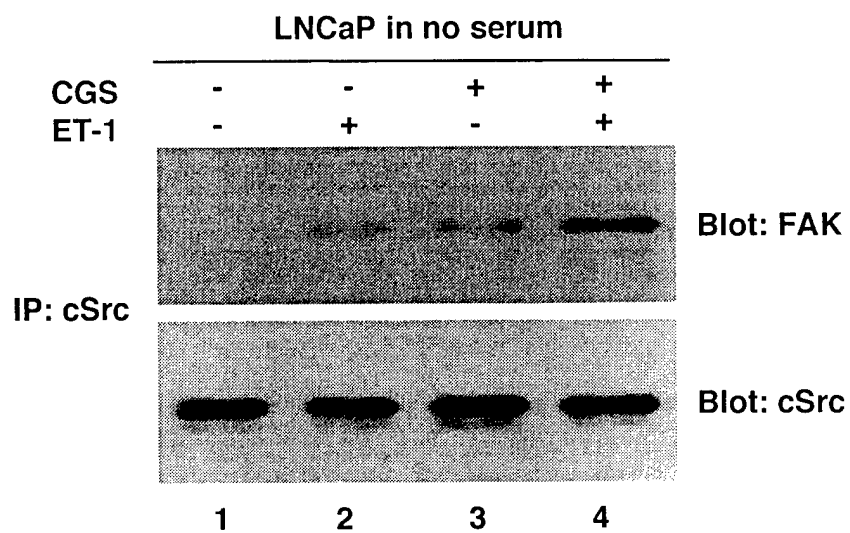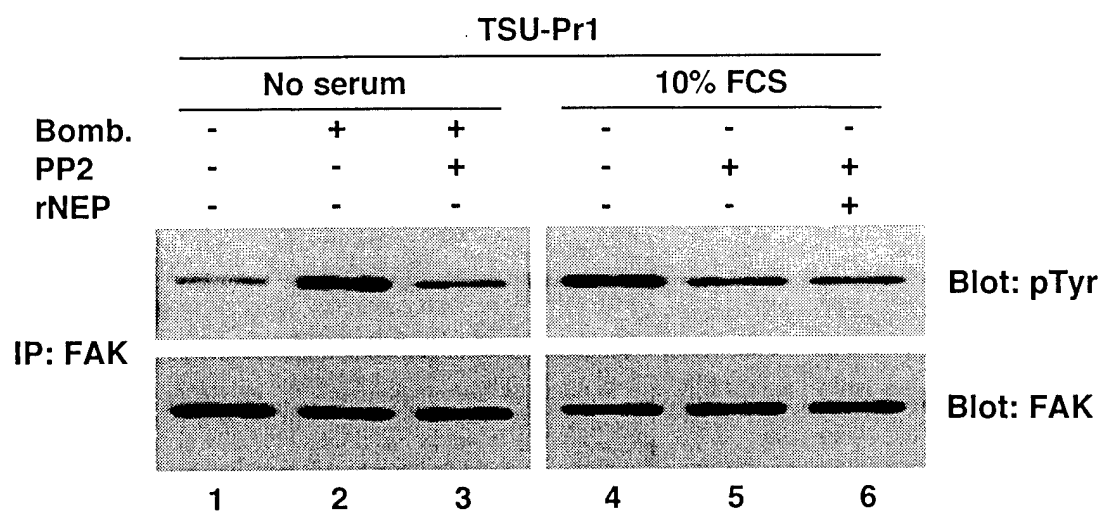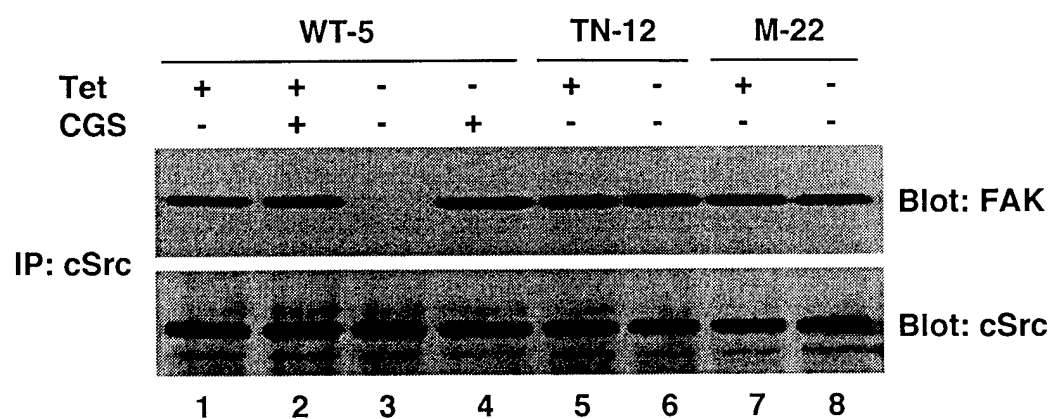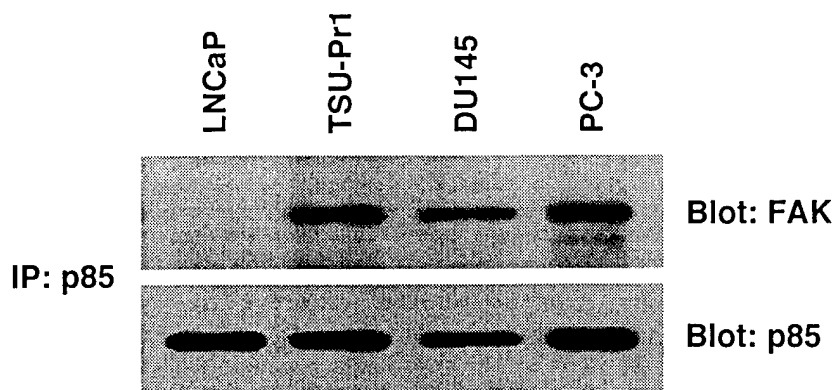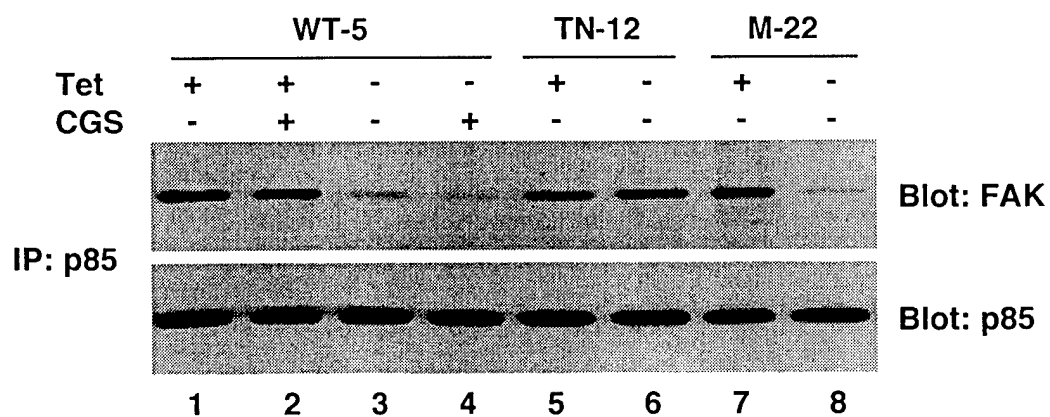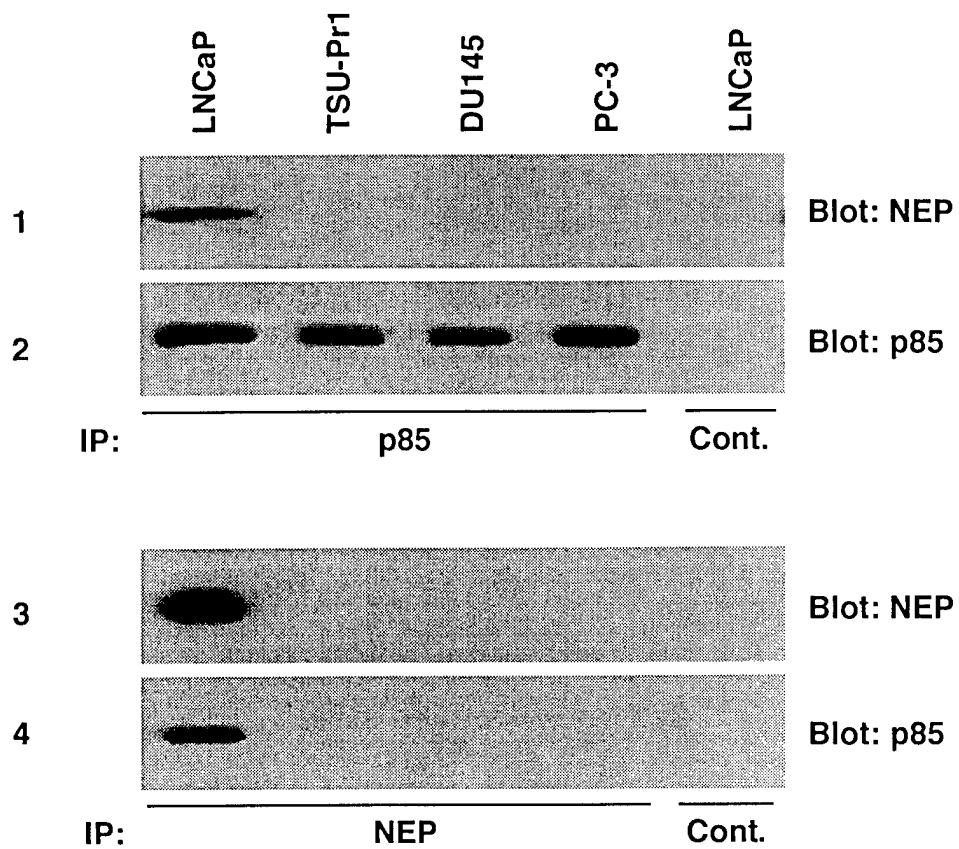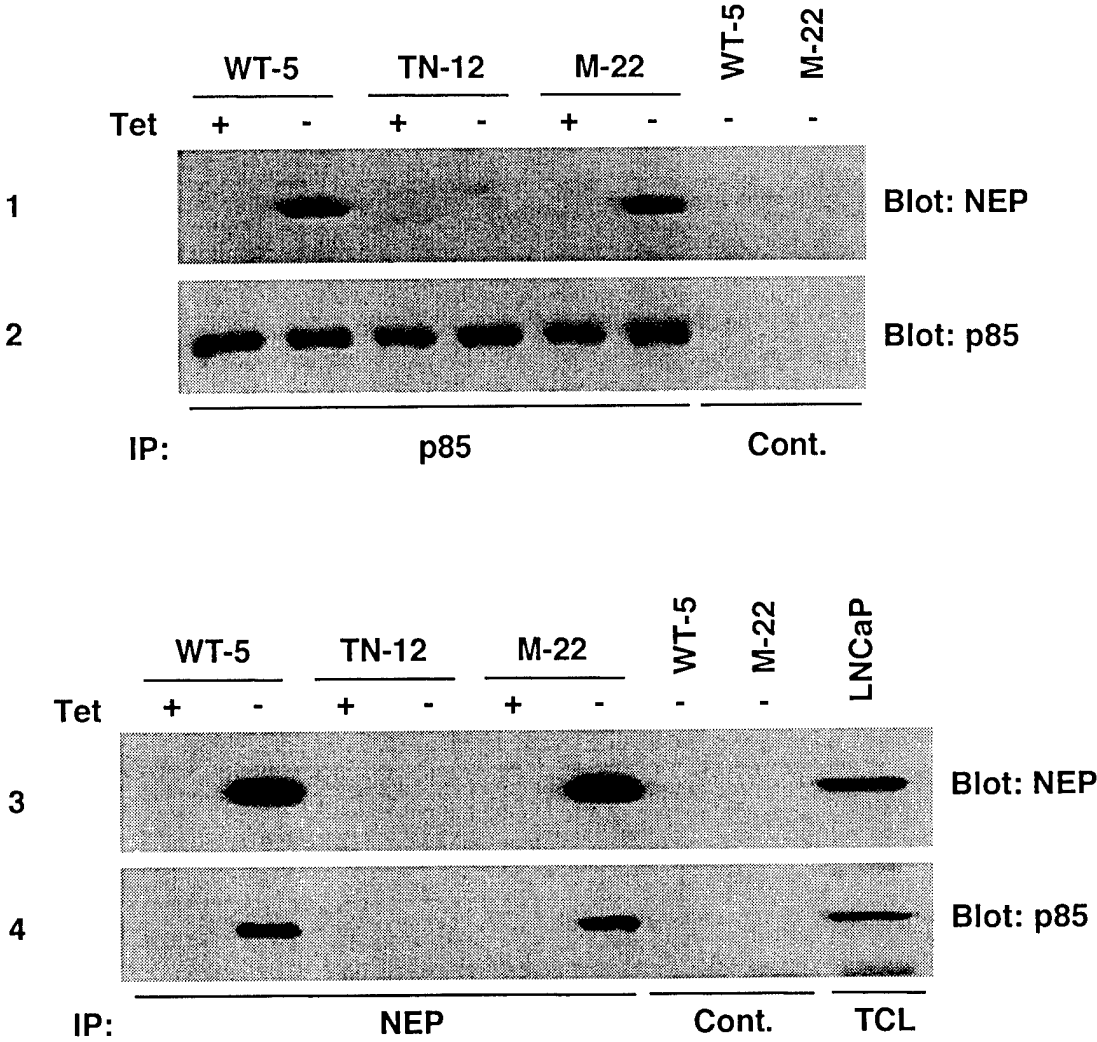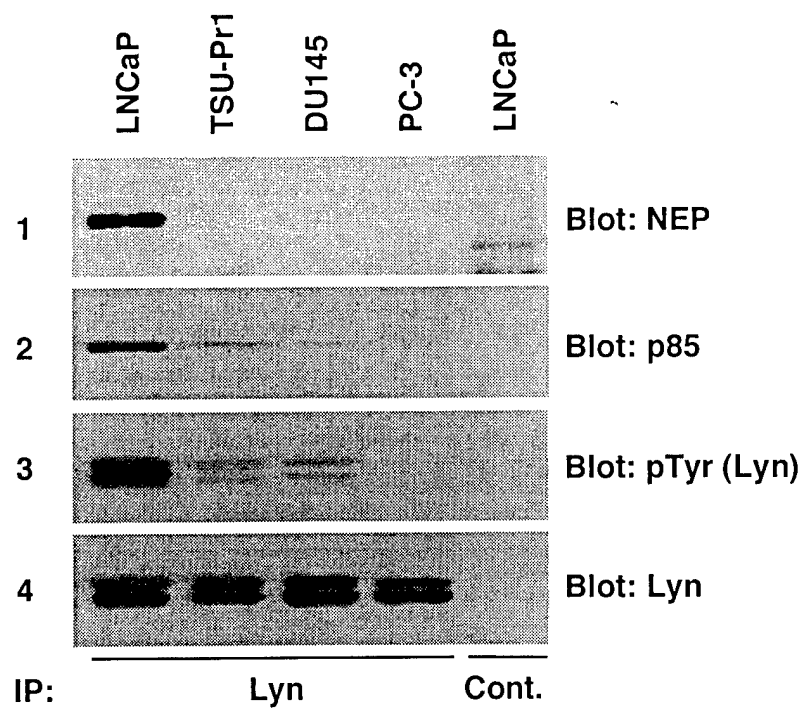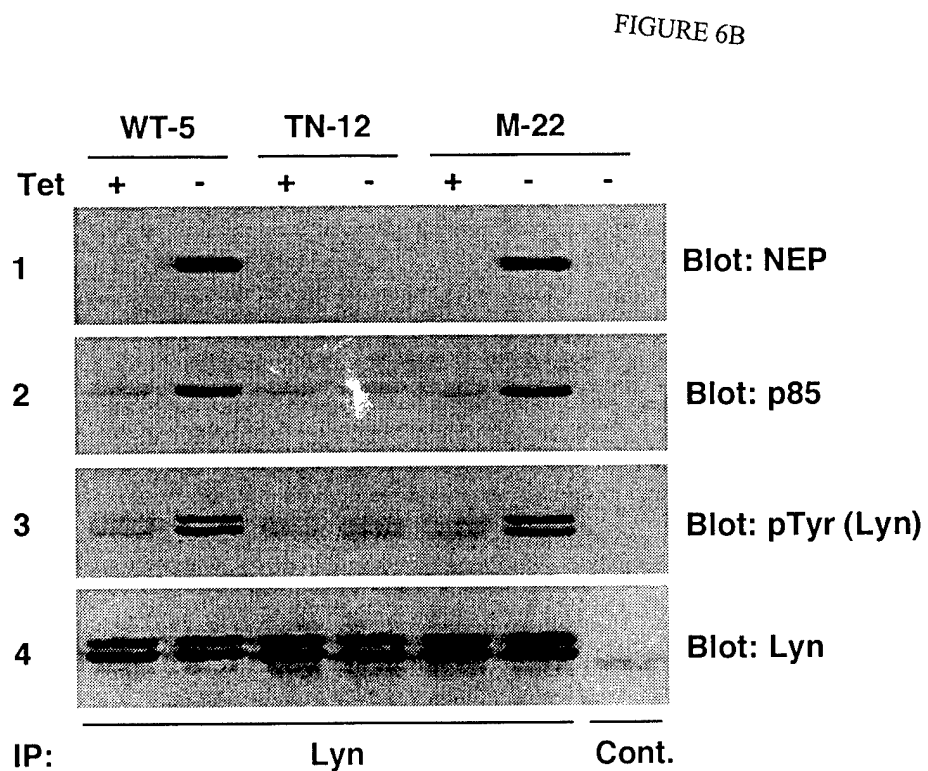
expressed only ERβ and PR mRNAs. Cell proliferation studies revealed that the growth of PC-3 cells was inhibited by the estrogen, 17β-estradiol (E₂), and antiestrogens, ICI and Tam, while that of DU145 was blocked only by the antiestrogens. These findings suggested that growth inhibitory actions of estrogens/antiestrogens on prostate cancer cells are dependent on the ER-subtypes expressed in the cells. Furthermore, exposure of DU145 cells to an ERβ-antisense-oligonucleotide rescued these cells from the ICI-induced growth inhibition, hence landing additional support to a role played by ERβ in antiestrogen action. (Supported by NIH award CA15776 and US Army DAMD17-98-1-8608).

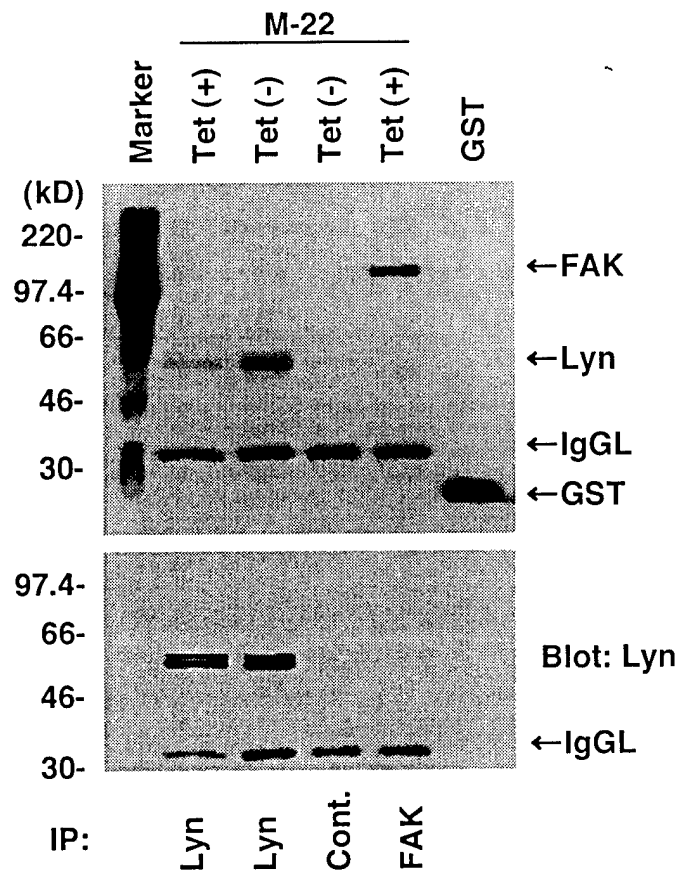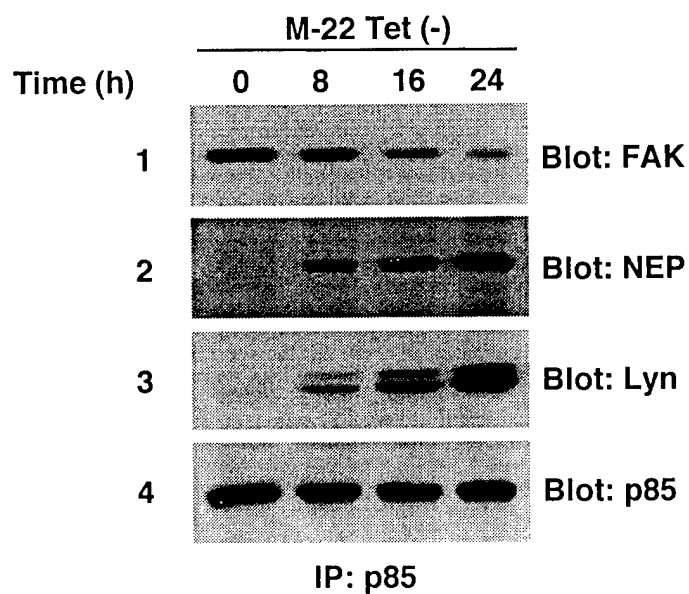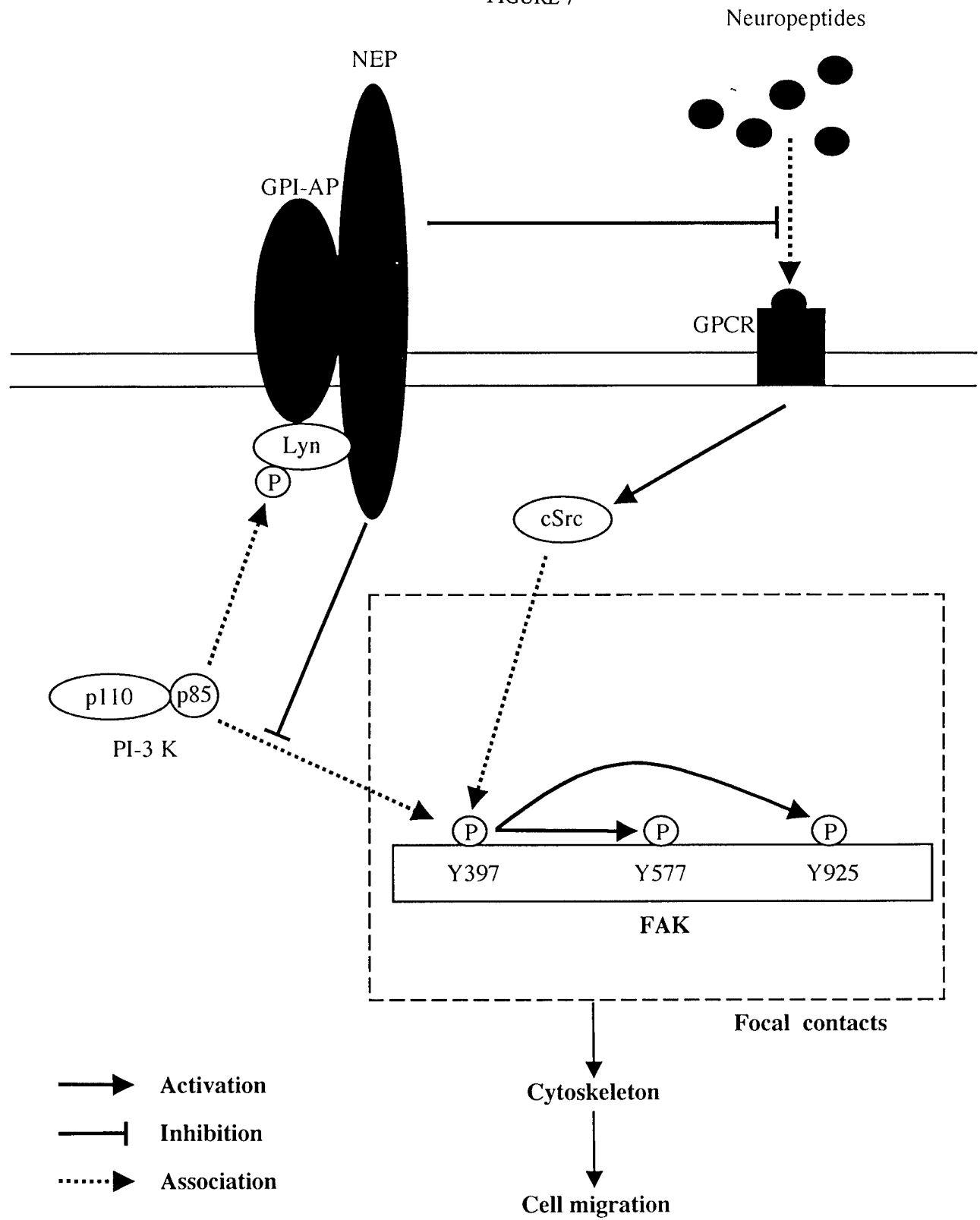**#4204   Effects of 1,25-dihydroxycholecalciferol (calcitriol) alone and in combination with dexamethasone or methylprednisolone on mitogen-activated protein kinase (MAPK) in murine squamous cell carcinoma (SCC) cells.** McGuire, T.F., Hershberger, P.A., Maisel, C.M., Trump, D.L., and Johnson, C.S. Departs. of Pharmacology and Medicine, University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213.

Calcitriol inhibits the growth of murine SCC cells in vitro and in vivo. These effects of calcitriol are significantly enhanced by co-treatment with dexamethasone (DEX) but not by co-treatment with methylprednisolone (MP). The mechanisms by which calcitriol exerts its growth inhibitory effects and DEX enhances the inhibitory effects of calcitriol are unclear. In vitro, exponentially growing SCC cells express high levels of phosphorylated/activated MAPK (erk1 and erk2), proteins which are known to transduce mitogenic signals to the nucleus in response to a number of extracellular stimuli. We have examined the effects of calcitriol alone and in combination with either DEX or MP on MAPK in SCC cells. Treatment with calcitriol (10 nM), DEX (0.5 μM), or MP (2.5 μM) alone only modestly decreased levels of tyrosine phosphorylated MAPK at 24 h. At 48 h, levels of activated MAPK were further decreased by calcitriol alone, while DEX and MP alone failed to decrease levels beyond that of control. Treatment with calcitriol and DEX resulted in a significant decrease in levels of activated MAPK at 24 h; by 48 h, levels were nearly undetectable as compared to calcitriol or DEX alone. In contrast, at 24 h and 48 h, treatment with calcitriol and MP did not significantly change levels of activated MAPK as compared to calcitriol or MP alone. These results suggest that calcitriol exerts its growth inhibitory effects on SCC cells by inhibiting the mitogenic signaling pathway at a point upstream of MAPK. Supported by NIH grant CA67267 and a grant from CaPCURE.

#4205   Tumor suppressive effect of neutral endopeptidase in androgen-independent prostate cancer cells. Dai, J., Shen, R., Geng, Y., Yang, M., Sumitomo, M., Powell C.T., Garzotto, M., and Nanus, D.M. Department of Urology, Weill Medical College of Cornell University, New York, New York 10021, and the Department of Urology, Memorial Sloan-Kettering Cancer Center, New York, New York 10021.

Neutral endopeptidase 24.11(NEP) is a cell-surface peptidase whose loss contributes to the development of androgen-independent prostate cancer (Nature Medicine 1998;4:50). To determine the effects on PC cells of overexpressing cell-surface NEP, we used the inducible tetracycline-regulatory gene expression system to introduce the NEP gene into androgen-independent Tsu-Pr1 cells. Cell proliferation over one week was >80% inhibited in induced NEP-expressing clones (WT5 and WT24) compared to control (N12) or M22 cells containing a mutated, catalytically inactive NEP gene. NEPs effect on tumor formation was examined by orthotopically injecting 1×10⁶ cells (WT5 and N12) into the prostate of athymic mice and the animals sacrificed at 30 days. No tumor were detected in NEP-induced WT5 inoculated prostates (ave. wt. 0.21 gm) vs. 100% of N12 inoculated prostates (ave. wt. 1.03 gm). Both NEP and the protein-tyrosine kinase inhibitor Genistein can inhibit phosphorylation of p125FAK (focal adhesion kinase) on tyrosine. Induced cell-surface NEP and exogenous recombinant NEP + Genistein resulted in a ~50% decrease in PC cell number over 3 days compared to Genistein alone (P<0.005) at a concentration 5–10 fold less than the IC50 for Genistein. Flow cytometric analysis shows that induced NEP expression in WT-5 cells results in a G2+M arrest. These data suggest that NEP may possess tumor suppressor function which can be developed into a novel therapy for androgen-independent prostate cancer.

#4206   Interactions of 1,25-dihydroxyvitamin D₃ (calcitriol) with androgens and anti-androgens in the regulation of prostate cancer cell growth. Murthy, S., Marcelli, M. and Weigel N.L. Baylor College of Medicine, Houston, TX 77030.

Growth inhibitory effects of calcitriol on androgen dependent LNCaP cells are reported to be enhanced in the presence of dihydrotestosterone (DHT), and antagonized by the anti-androgen, Casodex (Xhao et al. Endocrinology 138, pp.3290–98, 1997), both of which are individually growth inhibitory. Since LNCaP cells have a mutant androgen receptor (AR), the effects of calcitriol, DHT and Casodex were tested on the PC-3AR line, a PC-3 derived line stably expressing wild-type AR at levels comparable to LNCaP. Transactivation studies have shown that these cells have an active AR and that Casodex acts as an antagonist. DHT was found to enhance calcitriol mediated growth inhibition in the PC-3AR cells similar to the LNCaP, but antagonism by Casodex was not observed. Transactivation experiments using a luciferase reporter have shown that Casodex doesn't affect the ability of the Vitamin D receptor to activate transcription. LNCaP sublines that are androgen independent in vivo were also investigated. In contrast to LNCaP cells, these lines (LN3 and C4-2) were growth inhibited by calcitriol in

the absence of endogenous androgens. In the presence of FBS, these cell lines also exhibited a positive interaction between calcitriol and DHT, and a partial antagonism of calcitriol mediated growth inhibition by Casodex. These results indicate that while the functional interaction between calcitriol and DHT may be a feature of all AR+ prostate cancer lines, the antagonism of calcitriol by Casodex may be characteristic of LNCaP derived lines. The mechanism of these interactions is under further investigation.

#4207   Interaction of BAG-1p50 with the vitamin D receptor suppresses vitamin D-mediated inhibition of proliferation. Authors: Witcher, M., Yang, X., Pater, A., Tang, S.C.*. Memorial University of Newfoundland, St. Johns, NF, Canada.

BAG-1 is an anti-apoptotic protein with four isoforms (BAG-1p50, p46, 33 and p29) that has been found to be overexpressed in several types of human cancers. Human BAG-1p46 was originally isolated due to its ability to bind several members of the steroid hormone receptor superfamily. Because of this ability, we decided to investigate whether or not the recently isolated full length BAG-1 protein (BAG-1p50) could interact with the vitamin D receptor (another member of the steroid hormone superfamily) and whether this interaction is functionally significant. We found by Far Western blots and glutathione S-transferase -BAG-1p50 pull-down assays that BAG-1p50 could interact with the vitamin D receptor (VDR). Gel shift assays using cell extracts from BAG-1p50 stably transfected U87 glioblastoma cells showed that BAG-1 could inhibit the VDR from binding to its response element. Furthermore, overexpression of BAG-1p50 not only resulted in an increased rate of proliferation but rendered the cells resistant to vitamin D-induced growth inhibition. BAG-1p50 could also inhibit vitamin D-mediated gene expression as was evidenced by its blockage of vitamin D-induced transcriptional up-regulation. These results suggest that BAG-1p50 can participate in the vitamin D signal transduction pathway and aids in our understanding of vitamin D resistant cells and tumors.

#4208   BAG-1 and estrogen receptor function in breast cancers. Knee, D.A., Krajewski, M., Krajewska, M., Clevenger, C., Reynolds, C., and Reed, J.C. The Burnham Institute, La Jolla, CA 92037; U. Penn. Dept. Pathol. Phil., PA 19104.

BAG-1 is a Hsp70/Hsc70-binding protein that forms complexes with several nuclear hormone receptors and some other proteins At least three isoforms of BAG-1 are produced by alternative translation site selection within a common mRNA: BAG-1, BAG-1M (Rap46/Hap46), and BAG-1L. We observed that BAG-1 and BAG-1L can enhance Erα-mediated transactivation of an ERE-containing reporter gene. BAG-1 and BAG-1L enhance both ligand-dependent and ligand-independent transactivation by ERα and also render ERα more resistant to the effects of anti-estrogens, such as tamoxifen and ICI182,780. Stable over-expression of BAG-1 in ER-positive breast cancer cell lines, such as ZR-75-1, abrogated in vitro growth suppression by anti-estrogens. BAG-1 also increased resistance of MCF-7 cells to adriamycin in the presence of estrogen, consistent with reports that estrogen provides protection against this anticancer drug. Thus, BAG-1 family proteins may potentially contribute to treatment failures in breast cancer patients subjected to anti-estrogen therapy. Interestingly, immunohistochemical analysis of BAG-1 expression in primary breast cancers revealed a correlation between ER-positivity and nuclear BAG-1 immunostaining. The findings provide further evidence of a potentially significant functional interaction between BAG-1 and ER in breast cancers.

#4209   The histone acetyltransferase Tip60 is a co-activator of the human androgen receptor protein. Robson, C.N., Brady, M.E., Ozanne, D.M., Waite, I., Cook, S. and Neal, D.E. School of Surgical and Reproductive Sciences, Newcastle University, Newcastle upon Tyne, NE2 4HH, UK.

Prostate cancer is the most commonly diagnosed cancer in Western men. Prostatic tumors are initially androgen-sensitive, recurrent tumors are generally androgen-independent and resistant to treatment. Understanding the molecular mechanisms involved in androgen-independent growth is a key step in developing rational treatment approaches to the disease. Androgen effects are mediated through the androgen receptor (AR) which is a member of the nuclear hormone receptor superfamily of ligand dependent transcription factors. Our work has focused upon defining the mechanisms of transcriptional control exacted by the AR. Using a C-terminal region of the human AR in a yeast 2-hybrid screen, we have identified the histone acetyltransferase Tip60 as an AR interacting protein. Tip60, originally identified as a co-activator for the HIV TAT protein enhances AR mediated transactivation in a ligand dependent manner in LNCaP and COS-1 cells. Additionally, Tip60 enhances transactivation through the estrogen and progesterone receptors in a ligand dependent manner. Our studies demonstrate that Tip60 co-immunoprecipitates with AR and SRC-1 in vitro and that Tip60 enhances transactivation to levels observed with the co-activators SRC-1, p300 and CBP. The importance of such proteins in enhancing nuclear hormone receptor mediated transcriptional activation is widely accepted and this work suggests that Tip60 may play an equally important role.

#5525 PTEN NEGATIVELY REGULATES CELL ADHESION THROUGH DEPHOSPHORYLATION OF FOCAL ADHESION KINASE (FAK) AND PAXIL-LIN. Joerg Haier, and G. L Nicolson, Institute for Molecular Medicine, Huntington Beach, CA

The regulation of integrin-mediated cell adhesion and its stabilization involves different phosphorylation and dephosphorylation events, mainly on Tyr-residues. PTEN is a dual-specific phosphatase that can dephosphorylate Tyr, Ser and Thr. Focal adhesion kinase (FAK) was recently found to be a substrate of this phosphatase in glioma cells, where it appears to be involved in regulation of cell spreading and migration as part of focal adhesions. We have investigated the role of PTEN in cell adhesion of HT-29 human colon carcinoma cells to extracellular matrix under static and hydrodynamic conditions. PTEN coprecipitated with FAK and paxillin, and the amount of coprecipitation was dependent on the formation of adhesions to collagens. This corresponded with an adhesion-dependent increase in Tyr-phosphatase activity of PTEN. Using preparations of native FAK, paxillin and PTEN from HT-29 cells in a specific Tyr-phosphatase assay FAK and paxillin were identified as substrates for this dephosphorylation. Phenyl arsine oxide (PAO) was able to inhibit the activity of PTEN, whereas it did not affect another Tyr-phosphatase (PTP1B). PAO also completely inhibited cell adhesion under static conditions in a microtiterplate assay and under dynamic conditions in a laminar flow chamber, but treatment of cells attached to a substrate did not result in their detachment. In addition, this inhibitor was able to suppress adhesion-dependent Tyr-phosphorylation of FAK and paxillin and their coprecipitation with PTEN. In attached cells treatment with PAO resulted in an electrophoretic mobility shift of paxillin, but the amounts of phosphorylated FAK and paxillin were not affected. We conclude that PTEN appears to be involved in the regulation of integrin-mediated adhesion through dephosphorylation of FAK and/or paxillin. This phosphatase might play a role as a negative regulator for the formation of stable HT-29 cell adhesion to extracellular matrix.

#5526 SIGNALING TO INVASION IN HUMAN GLIOMA CELL LINES VIA PROTEIN KINASE C (PKC), EXTRACELLULAR-SIGNAL-REGULATED KINASE (ERK), AND ORNITHINE DECARBOXYLASE (ODC). A. B da Rocha, D. R A Mans, G. Lenz, R. Rodnight, and G. Schwartsmann, Fed Univ Rio Grande do Sul, Porto Alegre, Brazil, and Lutheran Univ Brazil, Canoas, Brazil

Matrix metalloprotease 2 MMP-2 and its activating enzyme membrane-type-1 metalloprotease (MT1-MMP) are critical to glioma invasion. The expression of these enzymes may be regulated via pathways involving the epidermal growth factor receptor (EGFR), Ras, ERK, and ODC. We previously showed the induction of ODC in human glioma cell lines through distinct PKC- and EGFR-mediated mechanisms, both acting via ERK. Recent data also implicate PKC in glioma cell invasion. In this study, we examined the roles of ERK and ODC in this action of PKC. Thus, A-172, U-87, and U-251 human glioma cells were treated with phorbol 12-myristate 13-acetate (PMA), GF 109203X. calphostin C, PD 098059, and/or DLα-difluoro-methylornithine (DFMO). Cells were then assessed for in vitro invasion. MMP-2 activity, and MT1-MMP mRNA, by their migration through Matrigel, zymography, and Northern blotting, respectively. PKC inhibition by 2 h GF 109203X 5 μM or calphostin C 40 nM led to a 2.5-fold decrease in these determinants in A-172 and U-87 cells. PKC stimulation by 30 min PMA 100 nM induced the opposite effects. The addition of PD 098059 20 μM or DFMO 5 mM alone or together, potentiated the inhibitory effects of GF 109203X and calphostin C about 2.0-, 1.5-, and 4.0-fold, respectively. Either treatment alone counteracted the stimulatory effects of PMA by about 40%, but both agents together reduced the PMA effects to control values. The PKC modulators did not affect MMP-2 activity and MT1-MMP MRNA in the U-251 cell line, partially explaining its poor invasiveness. Our data suggest an important role of the PKC-ERK-ODC pathway in MMP-2 activity and MT1-MMP expression and thus invasion in human glioma cell lines.

#5527 MODULATION OF NF-KB ACTIVATION REGULATES INTERLEU-KIN-8 EXPRESSION IN HUMAN MALIGNANT MELANOMA CELLS. Rakesh K Singh, Prabhudas S Patel, and Michelle L Varney, The Gujrat Cancer and Res Institute, Ahmedabad, India, and Univ of Nebraska Med Ctr, Omaha, NE

In the present study we demonstrate that up-regulation of IL-1β and TNF-α mediated IL-8 expression in malignant melanoma cells is regulated by the activation of transcriptional factor Nuclear Factor Kappa B (NF-κB). Treatment of melanoma cells with IL-1β and TNF-α significantly stimulated IL-8 expression in the cells. Addition of capsaicin (8-Methyl-N-Vanillyl-6-nonenamide), a known inhibitor of NFκB, resulted in inhibition of constitutive as well as IL-1β and TNF-α-induced IL-8 in melanoma cells. The effect of inhibition of IL-8 expression was dependent on concentration of capsaicin and duration of treatment. Further, electrophoretic mobility shift assay (EMSA) from nuclear extracts from melanoma cells showed a constitutive activation of NF-kBp50 and NF-kBp65. Melanoma cells treated with IL-1β showed enhanced NF-kBp50, but not p65 levels. However, treatment of melanoma cells with capsaicin inhibited activation both NF-kBp50 and p65. Further, modulated levels of IL-8 in capsaicin treated melanoma cells was associated with inhibition of in vitro proliferation. These results demonstrates that IL-8 up-regulation in melanoma cells by inflammatory cytokines is mediate through NFκB activation.

#5528 P38 MITOGEN-ACTIVATED PROTEIN KINASE REGULATES MMP-2 EXPRESSION AND INVASION OF MALIGNANT MELANOMA CELLS. Carsten Denkert, Antje Siegert, Anja Leclere, Andreas Turzynski, and Steffen Hauptmann, Charite Hospital, Berlin, Germany

Proteolytic enzymes, such as matrix-metalloproteinase (MMP)-2 are important factors for tissue invasion of malignant melanoma cells. The signal transduction pathways regulating expression of matrix-metalloproteinases are not completely understood so far. P38 mitogen-activated protein kinase (p38MAPK) is a member of the mitogen-activated protein-kinase family mediating cellular responses to environmental stress as well as inflammatory stimuli. Concerning similarities between inflammatory tissue and tumor tissue, we investigated the function of p38MAPK in invasion of the malignant melanoma cell line MeWo. To investigate the role of different mitogen-activated protein kinase signaling pathways in melanoma invasion and MMP expression, we used the specific p38MAPK inhibitor SB 203580 as well as the specific MEK-1 inhibitor PD 98059. Using immunoblotting as well as confocal microscopy, p38MAPK was found to be constitutively phosphorylated at low levels in MeWo cells. Inhibition of MMP-2 mRNA expression was found after treatment of cells with SB 203580 for 24h as well as 48h. Inhibition of mRNA levels could be observed using concentrations of SB 203580 as low as 1μM. Treatment of cells with PD 98059 did not change MMP-2 mRNA expression. Parallel to the changes observed in mRNA levels of MMP-2 there was a reduction of gelatinase activity of MMP-2 in gelatine zymography. Invasion of cells through a matrigel basement membrane was reduced by 60% after inhibition of p38MAPK, while inhibition of MEK-1 did not change matrigel invasion. This study shows that p38 MAPK regulates expression of MMP-2 and invasion of malignant melanoma cells in-vitro.

#5529 REGULATION OF MICROFILAMENT REORGANIZATION AND IN-VASIVENESS OF BREAST CANCER CELLS BY P21-ACTIVATED KINASE-1 K299R MUTANT. Liana Adam, Ratna K Vadlamudi, and Rakesh Kumar, UT MDACC, Houston, TX

To further explore the roles of p21activated kinase-1 (Pak1) in the invasive behavior of breast cancer cells, we investigated the influence of inhibition of Pak1 activity on the reorganization of cytoskeleton components that control motility and invasiveness of cells, using a highly invasive breast cancer MDA-MB435 as a model system. Our results demonstrate that overexpression of a kinase-dead K299R Pak1 mutant leads to suppression of motile phenotypes as well as invasiveness of cells both in the absence or presence of exogenous HRG. In addition, these phenotypic changes were accompanied by a blockade of disassembly of focal adhesion points, stabilization of stress fibers, and enhanced cell spreading in an extracellular matrix-independent manner. We also demonstrated that in K299R Pak1 expressing cells, F-actin filaments were stabilized by persistent co-localization with the actin-binding protein tropomyosin and caldesmon. Furthermore, inhibition of Pak1 activity in breast cancer cells was associated with a reduction in JNK kinase activity, inhibition of DNA-binding activity of transcription factor AP-1, and suppression of in vivo transcription driven by AP-1 promoter (known to be involved in breast cancer invasion). These findings suggest that Pak1 downstream pathways have a role in the development and maintenance of invasive phenotypes in breast cancer cells.

#5530 RHO GTPASES, THEIR EFFECTORS, AND BREAST CANCER PRO-GRESSION TO THE METASTATIC STATE. Delia M Brownson, Kathleen L Stauber, and Suranganie F Dharmawardhane, The Univ of Texas at Austin, Austin, TX

To investigate Rho GTPase signaling events during breast cancer progression to the metastatic state, a range of human breast cancer cell lines established at various stages of disease progression was used as a model system. As a first step, comparative analysis of protein expression in these cell lines was performed by western blotting using specific antibodies. We examined the differential expression of signaling molecules (such as Rac, Cdc42, PTEN, PI3-kinase) known to play a role in cell survival, adhesive, and migratory properties. Compared to non-metastatic breast cancer cell lines (T47D, ZR-75, Hs578t), invasive metastatic cell lines (MDA-MB-231 and MDA-MB-435) exhibited lower levels of PTEN (phosphatase and tensin homolog deleted on chromosome ten) and slightly higher levels of PI3-kinase (phosphatidylinositol 3-kinase). Although, all breast cancer cell lines tested displayed similar expression levels of Rac and Cdc42, we hypothesized that the relative activity of Rho GTPases may be important triggers regulating metastasis. Thus, the relative activity of PTEN was further investigated in the context of the phosphorylation status of its substrates (i.e. FAK, Shc, and PIP3 levels). These substrates have been implicated in the activation of Rac and Cdc42, which are known regulators of cell motility and focal complex assembly. Moreover, transfection of Hs578t cells with activated forms of Rac and Cdc42 enhanced motile and adhesive properties of this non-metastatic breast cancer line. Therefore, the activity of Rho GTPases, regulated by the relative contribution of upstream effectors, may be influential in the process of breast cancer metastasis.

#5531 NEUTRAL ENDOPEPTIDASE INHIBITS CELL MIGRATION BY IN-HIBITING FOCAL ADHESION KINASE PHOSPHORYLATION IN PROSTATE CANCER CELLS. Makoto Sumitomo, Ruoqian Shen, Yiping Geng, Jie Dai, Daniel Navarro, Beatrice Knudsen, and David M Nanus, Weill Med Coll of Cornell Univ, New York, NY

Neutral endopeptidase 24.11 (NEP), whose loss contributes to the progression of prostate cancer (PC) cells (Nat. Med. 4: 50, 1998), cleaves neuropeptides such as bombesin and endothelin-I which can activate focal adhesion kinase (FAK) and induce cell migration. We investigated whether NEP inhibits FAK phosphorylation and cell migration in PC cells. Treatment of TSU-Pr1 or PC-3 cells with rNEP (50 μg/ml) for 2 hours blocked bombesin (50 nM)- or endpthelin I (10 nM)-induced FAK phosphorylation. Using TSU-Pr1 tetracycline-repressive clones which express wild-type NEP (WT5), catalytically inactive NEP (M22) or control (TN12), induced expression of NEP resulted in the decrease in both FAK phosphorylation (WT5: 96% and M22: 71%) and cell migration (WT5: 88% and M22: 71%) compared with TN12 control. Although the specific NEP inhibitor CGS24592 (100 nM) completely blocked NEP enzyme activity in WT5 cells, it failed to completely restore FAK phosphorylation. Similar to WT5, cell migration was >80% inhibited in TSU-Pr1 expressing a dominant-negative FAK. These findings suggest that NEP inhibits FAK phosphorylation and that the loss of NEP may play a crucial role in stimulating invasive progression of PC cells.

#5532 P21-ACTIVATED KINASE 1 PROMOTES ANCHORAGE-INDEPEN-DENT GROWTH OF HUMAN CANCER CELLS. Ratna K Vadlamudi, Liana Adam, Mahitosh Mandal, and Kumar Rakesh, UT MDACC, Houston, TX

Stimulation of growth factor signaling has been implicated in the development of aggressive phenotype, and Pak1 activation in human epithelial cancer cells. To study the role of Pak1 kinase in the regulation of motility and growth of epithelial cancer cells, we developed stable cell lines overexpressing kinase-active T423E Pak1 mutant under an inducible Tet promoter or stably expressing kinase-active H83, 86L Pak1 mutant deficient in GTPase binding. Expression of both T423E and H83, 86L Pak1 mutants in epithelial cells were accompanied by increased cell motility without any apparent effect on the growth-rate of cells. However, expression of kinase-active Pak1 mutants resulted in a significant stimulation of anchorage-independent growth of cells in soft-agar. In addition, T423E Pak1 expressing cells exhibited a regulatable stimulation of MAPK, and a specific MAPK kinase inhibitor preferentially interfered with the ability of conditional Pak1 expression to support anchorage-independent growth in soft-agar. In brief, our findings unravel a new function of Pak1, i.e., promotion of anchorage-independent growth of human epithelial cancer cells.

#5533 MITOGEN-ACTIVATED PROTEIN KINASE INHIBITORS BLOCK THE ONCOSTATIN M INDUCED DETACHMENT OF A375 MELANOMA CELLS FROM MONOLAYER CULTURES IN VITRO. R E Ryan, VA Med Ctr and Mountain States Med Research Inst, Boise, ID

Oncostatin M (OSM) is a pleotropic cytokine that is reported to inhibit, as well as enhance tumor progression. In a previous study we established that OSM is able to induce the detachment of viable melanoma cells from monolayer cultures in vitro. In the current study, the mitogen-activated protein kinase (MAPK)/inhibitors, PD 8059 and U0126 were observed to block the OSM induced detachment of A375 melanoma cells in vitro. OSM treatment (15ng/ml) for 24 hrs induced a 736 % increase in detached A375 cell levels versus control cultures. Co-administration of PD 98059, or U0126 (5 μM) with OSM reduced detached cell levels to 317 % and 116 %, respectively, of control cell cultures. The MAPK inhibitors did not inhibit A375 cell growth under these assay conditions. PD 98059 and U0126 treated cultures (with OSM) incorporated 89 % and 90 % of control ^{3}H-thymidine levels, respectively, whereas, OSM treated cultures incorporated 75 % of control ^{3}H-thymidine levels. Administration of PD 98059 or U0126, 8 hrs after OSM treatment also inhibited cell detachment. After 24 hrs incubation with OSM, PD 98059 and U0126 reduced detached cell levels from 735 % (OSM alone) to 265% and 99 %, respectively, of control levels. The MAPK activator, anisomycin (1 μM), also induced A375 cell detachment after a 24 hr incubation, where detached cell levels were observed to be 260 % above control cultures. In addition, the protein kinase C inhibitor, bisindolylmaleimide (5 μM) inhibited A375 cell detachment induced by OSM. When administered 8 hrs after OSM treatment, detached cell levels decreased from 736 % (OSM alone) to 158 % of control levels. The ability of a protein kinase C inhibitor to block cell detachment suggests cross talk between the MAPK and protein kinase C signal pathways. The data also indicates that activation of tyrosine kinases is an important component of OSM mediated cell detachment. In addition, a sustained activation of the MAPK pathway appears necessary to elicit cell detachment.

#5534 A HIGHLY METASTATIC HUMAN PANCREAS CANCER CELL LINE OVEREXPRESSES TRKB RECEPTOR TYROSINE KINASE. Wayne Frederick, Tom Wang, Yongde Bao, Tienai Wu, Christiane J Bruns, Isaiah Fidler, Douglas B Evans, and Paul J Chiao, The Univ of Texas M D Anderson Cancer Ctr, Houston, TX

Pancreatic adenocarcinoma is the fifth leading cause of adult cancer mortality in the United States and liver metastases are the dominant site of treatment failure. Conventional radiation therapy and chemotherapy are ineffective at controlling liver metastases. To better understand the biology of pancreas cancer for designing novel therapy, various in vivo models were tested. Recently, Bruns et al., have reported the identification and isolation of a highly liver metastatic pancreas cancer cell lines, L3.6pl from its parental COLO 357 FG cells by repeated in vivo selection of liver metastases. To identify the genetic determinants for inducing liver metastasis phenotype, we performed differential screening of cDNAs using cDNA arrays. The gene expression profiles between L3.6pl and FG

cell lines are almost identical, except neurotrophin growth factors receptors (TrkB). Our analysis has shown that TrkB is overexpressed in L3.6pl cells and undetectable in Colo357 FG cells . The overexpression of TrkB in L3.6pl cells was further confirmed using western blot analysis. Our data are consistent with the reports that highly invasive pancreas cancer tissues overexpress the neurotrophin growth factors and their receptors (Trk), and suggest that overexpression of TrkB in pancreas cancer cells may play a key role in inducing the liver metastatic phenotype. It will be interesting to test the novel therapeutic designs that specifically inhibit the Trk receptors for reducing the metastasis of pancreatic cancer.

#5535 ANTI-HER-2/NEU MAB ICR12 INHIBIT THE MIGRATION AND IN-VASIVE CAPACITY OF THE HUMAN BREAST CARCINOMA CELL LINE SKBR3 AND ANGIOGENESIS. Helmout Modjtahedi, C J Dean, C Shotton, W Court, S Eccles, T Avades, and H Thomas, Institute of Cancer Res, Sutton, United Kingdom, and Univ of Surrey, Guildford, United Kingdom

The HER-2/neu (c-erbB-2) proto-oncogene encodes a 185-kDa transmembrane glycoprotein (p185[HER-2]) with tyrosine kinase activity which is a member of the type-I growth factor receptor family. Overexpression of p185[HER-2] has been reported in a number of human malignancies and has been associated with poor prognosis in many patients. Here, we investigated the effect mAb ICR12, which is directed against the external domain of HER-2, on the proliferation, migration and invasive capacity of the HER-2 overexpressing cell line SKBR3. We found that at 100nM, mAb ICR12 did not have any effect on the proliferation of SKBR3 cells in vitro. However, at the same concentration mAb ICR12 inhibited the migration of SKBR3 cells through a 24-well Transwell™ chamber with polycarbonate filters by 90% and decreased their invasive capacity through Matrigel by 93%. At the same concentration, anti-EGFR mAb ICR62 inhibited the growth, migration and invasive capacity of the EGFR overexpressing tumours, but did not have any effect on the growth, migration or invasive capacity of the HER-2 overexpressing SKBR3 cells. Using a human angiogenesis kit (TCS BIOLOGICALS), we found that ICR12 inhibited angiogenesis by 85%, as determined by staining with antihuman CD31 antibody. Our data suggest that the HER-2 blockade by mAb ICR12 has potential in inhibiting the metastatic capacity of HER-2 overexpressing tumours and may also be an effective inhibitor of angiogenesis.

MOLECULAR BIOLOGY 61: Tumor Suppressor Genes in Cancer: Correlation with Progressive Markers

#5536 ANALYSIS OF PPARγ GENE IN HUMAN MALIGNANCIES. Takayuki Ikezoe, Seiji Kawano, Elizabeth A Williamson, Junichi Hisatake, Eric Green, Carl W Miller, Hirokuni Taguchi, and H. Phillip Koeffler, Cedars-Sinai Med Ctr, Los Angeles, CA, Harvard Univ, Cambridge, MA, and Kochi Med Sch, Kochi, Japan

Peroxisome proliferator-activated receptor γ (PPARγ), which plays an important role in adipocyte differentiation, is expressed in many human malignancies, including those from prostate, breast as well as colon. It regulates differentiation and/or cell growth of these cells. The PPARγ gene has been mapped to chromosome band 3p25, where recurrent chromosomal abnormalities are seen in a variety of human malignancies. Therefore, PPARγ could be an important tumor suppressor gene. In fact, a recent study revealed that PPARγ gene is functionally mutated in sporadic colon cancer cells. This prompted us to investigate whether PPARγ gene is mutated in other cancer types. A total of three hundred and twelve samples, including prostate, breast and lung cancers and leukemia were analyzed for mutations of PPARγ gene by reverse transcriptase-polymerase chain reaction-single strand conformation polymorphism (RT-PCR-SSCP) or PCR-SSCP analysis. No shifted bands were detectable in any of the human malignancies.But we found that 21 of 23 Pre B-ALL clinical samples did not express transcript of PPARγ. In contrast, normal CD19-positive B-cells, CD3-positive T-cells, CD34-positive hematopoietic stem cells as well as 42 cell lines from a variety of cancers expressed PPARγ. Taken together, mutation of PPARγ gene is very rare in human malignancies except for colon cancer, but inactivation of PPARγ gene might play an important role in leukemogenesis of pre B-ALL.

#5537 CLONING OF THE PCDH9 GENE AT 13Q21, A NEW MEMBER OF THE PROTOCADHERIN GENE FAMILY DOWNREGULATED IN HUMAN BREAST CANCER CELL LINES. Jon T Bergthorsson, Ester Rozenblum, Don J D Weaver, Juha Kononen, Dietrich Stephan, Natalie Goldberger, Elizabeth Gillanders, Tommi Kainu, and Olli P Kallioniemi, National Human Genome Res Institute, Bethesda, MD, and Univ Hosp of Iceland, Reykjavic, Iceland

Breast cancers and several other tumors often show frequent somatic deletions distal to the Retinoblastoma locus at 13q14. A database search for candidate tumor suppressor genes identified a partial sequence of the protocadherin gene PCDH9 at 13q21.1. The protocadherins, like other members of the Cadherin superfamily are involved in cell adhesion. We therefore cloned a 4.6 kb PCDH9 transcript, which included the full 3 kb coding sequence, and studied its role as a candidate tumor suppressor gene. The predicted protein sequence of PCDH9 is most closely (41% identity) related to a Xenopus laevis NF-protocadherin, which has been implicated in cell to cell adhesion during embryogenesis. Other

upon the estrous cycle stage of tumor resection. All of the animals in proestrus at the time of resection had local recurrence, while those animals in estrus or metestrus had only 50% local tumor recurrence. The availability of a genetically marked breast cancer cell line, whose tumor biology is influenced significantly by the female reproductive cycle, is an invaluable tool for study of the mechanisms of this fascinating non-linear (cyclical) cancer biology.

#2728 DIFFERENTIAL STIMULATORY ACTIONS OF ESTRADIOL-17β-STEARATE ON THE GROWTH OF RAT MAMMARY VS. UTERINE CELLS.
Laura L Hook, Anthony J Y Lee, and Bao Ting Zhu, *Univ of South Carolina, Columbia, SC*

We hypothesize that the lipoidal estrogen-fatty acid esters may be particularly strong mitogens in the fat-rich mammary tissues compared with the uterus. To test this hypothesis, we compared the activities of 17β-estradiol (E_2) and estradiol-17β-stearate (E_2-17β-S) in stimulating the growth of mammary glandular cells vs the growth of uterus in ovariectomized Sprague-Dawley rats. Experimentally, 5 nmol of E_2 or E_2-17β-S were released daily to ovariectomized Sprague-Dawley rats for 10 days with an Alzet pump implanted under the back skin of the animal. Two hours before sacrifice, the animals received an i.p. injection of BrdU (50 μg/g body weight). The growth-stimulatory effect of E_2 and E_2-17β-S on mammary glandular cells was determined according to the BrdU-labeling indices, and their effect on the uterus was determined according to the uterine wet weight. The BrdU-labeling index for the mammary glandular cells of control ovariectomized rats was 1.7 ± 0.6%, whereas the labeling indices for E_2 and E_2-17β-S were increased to 4.1 ± 1.7% (2.4-fold) and 11.1 ± 1.6% (6.5-fold), respectively. In contrast, the growth-stimulatory effects of E_2 and E_2-17β-S in the uterus as determined based on uterine wet weight were not significantly different from each other. The data of our present study revealed that E_2-17β-S has a selective, strong hormonal effect in the fat-rich mammary tissues which was not observed with E_2.

#2729 INCREASED EXPRESSION OF NEUTRAL ENDOPEPTIDASE CAUSES ANDROGEN-MEDIATED GROWTH INHIBITION OF ANDROGEN RECEPTOR EXPRESSING ANDROGEN-INDEPENDENT PROSTATE CANCER CELLS.
Shen Ruoqian, Sumitomo Makoto, Dai Jie, Harris Adam, Burnstein L Kerry, and Nanus Michael David, *Univ of Miami Sch of Medicine, Miami, FL, and Weill Med Coll, Cornell Univ, New York, NY*

Androgen-induced growth repression of androgen-independent prostate cancer cells has been reported in androgen-independent PC-3 cells overexpressing the androgen receptor. We investigated whether androgen-induced expression of neutral endopeptidase 24.11 (NEP), a cell-surface peptidase which cleaves and inactivates neuropeptides implicated in the growth of androgen-independent prostate cancer, could contribute to the observed androgen-induced growth repression in PC3/AR. The addition of dihyrotestosterone (DHT) resulted in a ~30-40% decrease in cell number over 7 days concomitant with a significant increase in NEP enzyme specific activity. Northern analysis detected the high expression of androgen receptor and an increase in NEP transcripts following DHT treatment in PC3/AR cells. The same phenomenon was observed in an androgen-independent derivatives of LNCaP cells (LNCaP-OM1) which were obtained by chronically deprivation of androgen and expressed androgen receptor. The addition of the NEP enzyme inhibitor phospharamidon or the NEP competitive inhibitor CGS 24592 blocked the increase in NEP enzyme activity and reversed the DHT-induced growth inhibition. Neither phospharamidon nor CGS 24592 alone inhibited cell growth. These data indicate that androgen-induced growth repression of androgen-independent prostate cancer cells results in part from androgen-induced expression of NEP in these cells.

#2730 TESTOSTERONE PROMOTES SURVIVAL OF PROSTATE CANCER CELLS THROUGH UPREGULATION OF CAVEOLIN-1 AND CAVEOLIN-1 IS HIGHLY EXPRESSED IN ANDROGEN RESISTANT HUMAN PROSTATE CANCER.
Likun Li, Guang Yang, Chengzhen Ren, Jianxiang Wang, Michael M Ittmann, Thomas M Wheeler, Terry L Timme, Salahaldin Tahir, and Timothy C Thompson, *Baylor Coll of Medicine, Houston, TX*

Previously we demonstrated that caveolin-1 was associated with prostate cancer metastasis and that suppression of caveolin-1 expression re-established androgen responsiveness in prostate cancer cells. In this study we place caveolin-1 downstream of testosterone in a molecular pathway that leads to survival in mouse and human prostate cancer cells *in vitro*. Testosterone was shown to induce caveolin-1 gene expression at the level of transcriptional activation. In addition, antisense caveolin-1 was able to significantly inhibit the survival effects of testosterone indicating that caveolin-1 is a downstream effector of testosterone-mediated survial activities. Furthermore, adenovirus-mediated caveolin-1 expression alone was also shown to account for a significant component of the survival activities induced by testosterone *in vitro*. Immunohistochemical studies demonstrated a significantly increased frequency as well as distant metastases following androgen ablation therapy. Overall, our studies establish the testosterone-caveolin-1 pathway and caveolin-1 expression as important determinants of the androgen-resistant phenotype in prostate cancer.

#2731 ANDROGENS INHIBIT CAMA-1 BREAST CANCER CELL PROLIFERATION AND UP-REGULATE THE CYCLIN-DEPENDENT KINASE INHIBITOR P27^{KIP1}.
Jacques Lapointe, and Claude Labrie, *Oncology and Molecular Endocrinology Res Ctr, Quebec, PQ, Canada*

The androgen receptor is expressed in approximately 50% to 90% of breast tumors and androgens are known to inhibit the proliferation of breast cancer cells *in vitro* as well as *in vivo*. However, there are relatively few androgen-sensitive breast cancer cell lines available and the molecular mechanism of androgen-induced growth arrest remains unknown. We previously found that androgens down-regulate bcl-2 and up-regulate UDP glucose dehydrogenase expression in ZR-75-1 breast cancer cells. We report here that the androgen 5α-dihydrotestosterone (DHT) inhibits the proliferation of estrogen-sensitive CAMA-1 breast cancer cells. This inhibition of cell proliferation was dose-dependent and a maximal inhibition of estradiol-stimulated proliferation was observed at the concentration of 1 nM DHT. DHT-induced growth arrest was accompanied by an increase in the proportion of cells in the G1 phase of the cell cycle. To better understand the mechanism of DHT action we examined the expression levels of cell cycle regulators in control and DHT-treated cells. Compared to control cells, DHT-treated cells showed an increase in the relative proportion of hypophosphorylated retinoblastoma protein consistent with G1-arrest. We then examined the mRNA and protein levels of cyclin-dependent kinase inhibitors (CKIs). DHT-treated CAMA-1 cells showed increased levels of p27^{Kip1} mRNA and protein levels. These results suggest that inhibition of breast cancer cell proliferation by androgens may be mediated, at least in part, by an increase in p27^{Kip1} levels.

CELL AND TUMOR BIOLOGY 26: Metastatic Genes

#2732 REGULATION OF AP-2 EXPRESSION IN BREAST CANCER CELL LINES.
Okot Nyormoi, Janet E Price, Christina Roth, Reinhard Buettner, and Menashe Bar-Eli, *Inst fur Pathology Klinicum der Univ Regensburg, Regensburg, Germany, Univ Hosp RWTH, Aachen, Germany, and Univ of Texas M D Anderson Cancer Ctr, Houston, TX*

The purpose of this project was to investigate the regulation of activating protein 2 (AP-2) expression in breast cancer cells. AP-2 was shown to be over-expressed in cells that over-express HER2 and also to up-regulate HER2 in breast cancer cells. It is, therefore, important to understand the regulation of AP-2 expression in breast cancer cells. In this regard, the gene for an AP-2 repressing protein (AP-2rep), recently renamed krupple-like finger 12 (KLF12) was cloned. To determine whether KLF12 regulates AP-2 expression in breast cancer, we screened several breast cancer cell lines for AP-2 and KLF12 expression by semi-quantitative RT-PCR. Results showed that cells which express high level of AP-2 express low level of KLF12 and vice versa. This observation led us to investigate whether KLF12 modulates tumor progression by injecting cells that express high level of AP-2 and low level of KLF12 (9D3S), and low level of AP-2 and high level of KLF12 (9B2T) into mammary fat pads of nude mice. Both 9D3S and 9B2Tcell lines were derived from ZR75-1 mammary carcinoma cell line by limiting dilution. Results show that the rate of tumor growth (measured by tumor size) is directly correlated with the level of AP-2 and inversely correlated with that of KLF12 expression. From these observations, we conclude that AP-2 and KLF12 may significantly regulate the progression of breast cancer. Supported by NIH Grant CA77055-01 to ON.

#2733 INHIBITION OF TUMOR GROWTH AND METASTASIS OF HUMAN MELANOMA BY INTRACELLULAR ANTI-ATF-1-SINGLE-CHAIN FV FRAGMENT.
Carmen Tellez, Didier Jean, Suyun Huang, Darren W Davis, Steve H Hinrichs, and Menashe Bar-Eli, *Hmb-173, Houston, TX, Nebraska Med Ctr, Omaha, NE, and U T M D Anderson Cancer Ctr, Houston, TX*

Activating transcription factor-1 (ATF-1) and cAMP-responsive element (CRE)-binding protein (CREB) have been implicated in cAMP and Ca2+-induced transcriptional activation. The expression of the transcription factors CREB and ATF-1 is upregulated in metastatic melanoma cells. However, how overexpression of ATF-1/CREB contributes to the acquisition of the metastatic phenotype remains unclear. We have previously demonstrated that quenching of CREB/ATF-1 in metastatic melanoma cells by a dominant-negative CREB (KCREB) inhibited their tumorigenic and metastatic potential in nude mice and rendered them susceptible to apoptosis thus, indicating that ATF-1/CREB act as survival factors for melanoma cells (J. Biol. Chem; 273:24884, 1998). Here, the effect of disrupting ATF-1 activity was investigated using intracellular expression of an inhibitory anti-ATF-1 single chain antibody fragment (ScFv). Intracellular expression of SvFv anti-ATF-1 in MeWo melanoma cells caused significant reduction in CRE-dependent promoter activation and inhibition of ATF-1 and CREB binding to CRE DNA on EMSA gels. In addition, expression of ScFv anti-ATF-1 in melanoma cells suppressed their tumorigenicity and metastatic potential in nude mice. ScFv anti-ATF-1 rendered the melanoma cells susceptible to thapsigargin-induced apoptosis *in vitro* and caused massive apoptosis in tumors transplanted subcutaneously into nude mice. These studies demonstrate the potential of ScFv anti-transcription factor as a novel modality for cancer therapy.

#2365   SUSTAINED ACTIVATION OF MAPK IS REQUIRED FOR ESTRO-GEN DEPENDENT MALIGNANCY OF MCF-7 CELLS. Venkateshwar G Kesha-mouni, and Kaladhar B Reddy, *Wayne State Univ, Detroit, MI*

Estrogen and growth factors play a major role in normal development and tumor progression of breast. Earlier studies show that activation of MAPKinase (MAPK) may play a critical role in cross-talk between estrogen and growth factor mediated signaling. MAPK activation could be either a transient process or sustained depending on the external stimuli, cell type, and the physiological response. In addition it was shown that sustained activation of MAPK is required for cell motility and protease induction which play a role in tumor progression. In the present study using MCF-7 cells we show that TGF-α induces a rapid and transient activation of MAPK in 15 min and fall to control levels by 2 h. In contrast, estrogen induces a slow activation of MAPK which peaks at 4h and sustains upto 24 h. This sustained activation of MAPK by estrogen can be blocked by anties-trogen ICI-182,780 or RNA-synthesis inhibitor cycloheximide suggesting it is ER dependent and requires neosynthesis of proteins. The overexpression of EGF receptors in MCF-7 cells by stable transfection did not alter MAPK activation profile with TGF-α. Whereas, a monoclonal antibody against EGFR partially blocks the estrogen induced MAPK activation suggesting a autocrine stimulation, independent of receptor numbers. In addition we observed that both TGF-α and estrogen induce cell proliferation in MCF-7 cells. However only estrogen supports the anchorage independent growth of these cells in soft-agar which can be inhibited by PD9805 a MAPK inhibitor. Above results suggest that transient activation of MAPK mediates cell proliferation but sustained activation of MAPK may be a requirement for anchorage independent growth. This may account partly for the estrogen dependent tumorogenesis of MCF-7 cells.

#2366   ANTAGONISTIC ACTIONS OF TGF-β ON GLUCOCORTICOID RE-CEPTOR SIGNALING THROUGH C-JUN/AP-1. Sumudra Periyasamy, and Ed-win R Sanchez, *Med Coll of Ohio, Toledo, OH*

Cross-talk between the glucocorticoid receptor (GR) and transforming growth factor-β (TGF – β) signaling pathways has been examined because of their mutual involvement in the regulation of cell growth, development and differentiation. TGF- β antagonized dexamethasone (Dex)-mediated growth suppression in mouse fibrosarcoma L929 cells. TGF-β also repressed GR-mediated reporter (pMMTV-CAT) gene expression in a concentration dependent manner, with an IC_{50} of 5 ng/ml of TGF-β. Maximal inhibition (76%) was observed at 10 ng/ml of TGF-β. Conversely, Dex inhibited TGF-β-mediated promoter (p3TR-Lux) activity in these same cells. As TGF-β inhibition of GR-mediated gene expression oc-curred after Dex-mediated nuclear translocation of GR, we concluded that TGF-β inhibition of GR signaling occurs at the level of GR-mediated transcription. However, TGF-β did not repress GR-mediated gene expression using the pGRE2E1B-CAT minimal promoter construct, suggesting that the TGF-β inhibi-tion of GR signaling requires DNA binding factor(s) distinct from GR. We hypoth-esized that AP1, a transcription factor composed of c-jun and c-fos proteins, might be involved in the TGF-β inhibition of GR functions. Curcumin, a potent inhibitor of c-jun/AP1 expression, completely abolished the inhibitory effect of TGF-β on GR-mediated gene expression without affecting GR activity in the absence of TGF-β. These findings clearly demonstrate the existence of mutual antagonistic cross-talk between the GR and TGF-β by a mechanism involving AP-1.

#2367   BOMBESIN STIMULATES ANDROGEN RECEPTOR MEDIATED GENE TRANSCRIPTION. Jie Dai, Roslyn Stahl, Ruoqian Shen, Makoto Sumi-tomo, Daniel Navarro, Jong Bahk, Marvin Gershengorn, and David N Nanus, *Weill Med Coll of Cornell Univ, New York, NY,*

Neuropeptide growth factors such as bombesin and endothelin-1 are impli-cated in androgen-independent prostate cancer (AIPC) growth. To assess whether bombesin can enhance androgen receptor (AR) mediated gene expres-sion, we co-transfected the AR expression vector and the probasin promoter ARR3 tk-luc reporter into Swiss 3T3 and PC-3 cells, both of which express high affinity bombesin receptors. Dihydrotestosterone (DHT) increased luc transcrip-tion 7.9 and 11.8-fold at doses of 1 and 10 nM but had no effect at 10 pM. Bombesin (up to 50 nM) had no effect on luc transcription when added alone. The addition of bombesin to 1 or 10 nM of DHT did not further increase transcription. However, 5 nM bombesin + 10 pM DHT, doses that by themselves had no effect, resulted in a ~10 fold increase in transcription (p<0.005). This synergistic effect can be blocked by bombesin receptor antagonists and recombinant neutral endopeptidase which hydrolyzes bombesin. Bombesin + DHT also increases the binding of nuclear extracts from 3T3 and PC3 cells transfected with AR expres-sion vector to a consensus androgen response element in mobility shift assays, and increases the level of secreted prostate specific antigen (PSA) in the super-natant of LNCaP cells compared to DHT or bombesin alone. Immunoprecipitation of AR from LNCaP cells labeled with ^{32}P reveals that 5 nM bombesin + 10 pM DHT induces AR phosphorylation comparable to 1 nM DHT, while bombesin or 10 pM DHT alone do not. These data indicate that bombesin can synergize with low (castrate) levels of DHT to induce AR mediated transcription, and suggest that neuropeptides can promote AR mediated signaling in AIPC.

#2368   REGULATION OF ESTROGEN-INDUCED GROWTH BY TGF-β IN AN ERBB2 AMPLIFIED BREAST CANCER CELL LINE. Trevor A Petrel, Melanie A Lynch, Gary D Stoner, Christopher M Weghorst, and Robert W Brueggemeier, *The Ohio State Univ, Columbus, OH*

Breast cancer is hypothesized to advance from a hormone-responsive, estro-gen receptor positive (ER+) stage to a more aggressive hormone-independent, (ER-) stage by currently unknown mechanisms. Recent findings of interactions between powerful growth stimulating and suppressing pathways within the breast epithelial cell line, BT474, may provide a useful model for studying this progres-sion. TGF-β is a naturally occurring protein, which acts as a potent inhibitor of normal breast epithelial cell proliferation. The biological effects of TGF-β are mediated by a heteromeric receptor system (TβRII and TβRI) which classically inhibits normal cell growth by altering cell cycle components. The ER(+) breast cancer cell line, BT474, was not growth-arrested by TGF-β as determined by FACS analysis and BrdU incorporation into DNA. Although there was no effect by TGF-β on cell growth, ER protein levels were reduced by TGF-β to 50% of untreated control. The reduced ER was supported by decreased E2-mediated DNA synthesis indicating a potential role for TGF-β in tumor progression. Thus, in this system, cells can grow in the presence of TGF-β while ER levels are being reduced. Further studies explore the mechanisms responsible for TGF-β control of ER expression and its uncoupling to cell growth regulation in the hopes of gaining new insight into basic mechanisms responsible for progression of hor-monally-driven cancers. This research is supported by NIH CA66193 and NIH CA73698.

#2369   MITOGENIC AND PHENOTYPE-RELATED ACTIONS OF ENDO-THELIN-1 (ET-1) AND -3 (ET-3) IN HUMAN MELANOMA CELLS *IN VITRO*. Raffaele Tecce, Anna Bagnato, Marco Mangone, Alessandro Ranazzi, and Bar-bara Olivelli, *Regina Elena Cancer Inst, Rome, Italy*

We have previously reported that both ET-1 and ET-3 possess mitogenic activity in human melanoma cells *in vitro*, and that phosphorylation and activation of Erk-2 kinase is related to the activation of tyrosyne phosphorylation pathways involving phosphorylation/activation of Shc adaptor and pp125FAK kinase. We now report further observations on signal transduction pathways activated by endothelins in melanoma cells. First, as previously reported in astrocytes by Cazaubon and coworkers, ET-induced Shc and pp125FAK phosphorylation are differently regulated in the presence of cytochalasin: only the former is main-tained, while the latter is quite completely abolished. Furthermore, we have observed, in the melanoma cell line 2/60 expressing EGF-R, that ET-3 at con-centrations ranging between .1-1x10^{-7}M, besides inducing Shc phosphorylation determines as well EGF-R phosphorylation and transactivation, as previously observed by us in the ovarian carcinoma model in response to ET-1. Studies are under way in order to characterize the ET receptor subtype involved in this transactivation event, since usually ETB receptor subtype, associated to a per-tussis toxin-sensitive G-protein is preferentially expressed in melanoma cells, while ETA receptor, associated to a pertussis toxin-insensitive G-protein, is responsible for EGF-R transactivation in ovarian carcinoma. Finally, we have observed that exposure to ET-3 determines major variations, as determined by RT-PCR, in the levels of mRNA for surface antigens that are considered important in the biology of melanocytic lineage, such as ICAM-1, thus suggesting a role for endothelins in the physiology of normal and transformed melanocitic cell. Sup-ported by AIRC and Ministero della Sanità.

#2370   ASSOCIATION OF AIB1 WITH ESTROGEN RECEPTOR-α IN BREAST AND OVARIAN CANCER CELL LINES CONTAINING AIB1 AMPLIFI-CATION. David O Azorsa, Heather E Cunliffe, and Paul S Meltzer, *National Human Genome Res Institute, Bethesda, MD*

The steroid coactivator AIB1 (amplified in breast cancer-1) is a transcriptional coactivator which has been found to be amplified in about 9.5% of breast cancers and 7.4% of ovarian cancers. A role for the AIB1 protein in AIB1 gene amplified cell lines was investigated. Newly developed monoclonal antibodies (mAbs) were used in several assays to show an association between AIB1 and Estrogen Receptor-α(ER-α). AIB1 was shown to be more highly expressed in ER-α positive cell lines than in ER-α negative cell lines by Western blot analysis. Moreover, both AIB1 and ER-α co-localized to the nucleus of ER-α positive cell lines as shown by immunofluorescence microscopy. AIB1 was shown to be functionally associated with ER-α in gel retardation assays using the Estrogen Receptor Response Element (ERE). Labeled ERE was shifted by nuclear extracts from AIB1 amplified cell lines and were also supershifted using an anti-AIB1 mAb. Furthermore, anti-AIB1 mAbs were able to immunoprecipitate ER-α from lysates of chemically cross-linked cells but not from uncross-linked cells. These results suggest that the AIB1 and ER-α do associate physically in breast and ovarian cancer cell lines which are ER-α positive and this association may play a crucial role in the biology of these cells particularly in those which contain the AIB1 gene amplification.

#2371   ACETYLATION REGULATES ESTROGEN RECEPTORα TRANSAC-TIVATION. Chenguang Wang, Maofu Fu, Ruth Hogue Angeletti, Linda Siconolfi-Baez, Benita Katzenellenbogen, Vasily Orgryzko, and Richard G Pestell, *National Institute of Child Health and Human Development, Bethesda, MD, The Albert Einstein Cancer Ctr, Albert Einstein Coll of Medicine, Bronx, NY, and Univ of Illinois and the Coll of Medicine, Urbana, IL*